

ASPECTS OF THE DEVELOPMENTAL BIOLOGY OF OVARIAN FOLLICLES.

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ABSTRACT

Ovarian follicles are recruited from a pool of nongrowing primordial follicles and progress slowly through early phases of growth until the formation of an antrum. Little is known at present about the factors controlling follicle recruitment, preantral growth, or thecal and antral formation. This thesis reviews some aspects of early folliculogenesis and describes the development of a culture system for *in vitro* investigation of preantral growth. A enzymatic-mechanical isolation procedure was developed which permitted large numbers of isolated small ovarian follicle to be obtained for CBA/C57Bl6 hybrid mice, aged 8-10 days. The ovarian follicle is a spherical structure containing initially two main cell types - the oocyte and granulosa cells - with the thecal cells differentiating once the follicle has entered its growth phase. Interactions between these cell types may have an important role in controlling folliculogenesis. Cell shape, cell-cell interactions and cell-extracellular matrix interactions may all influence follicular growth. To support and maintain cell shape and the spatial relationships between the cell types in the ovarian follicle a culture system involving embedding the follicles within a three-dimensional collagen gel matrix was developed. A quantitative study of isolated murine follicle growth in collagen gel culture over two weeks was carried out. The system facilitated follicle growth from small to multilaminar stages but antrum formation and theca differentiation was not initiated. Cultured follicles were transferred under the kidney capsule of ovariectomized hosts. *In vivo* the follicles were able to progress to the Graafian stage. The effects of hormones and growth factors (oestrogen, follicle stimulating hormone and epidermal growth factor) on *in vitro* follicle development were studied using autoradiography and computer assisted image analysis.

Declaration

I declare that this thesis is of my own composition. The work described within this thesis was of my own devising and I was the main contributor to all phases of the work.

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TABLE OF CONTENTS

	<u>PAGE</u>
<u>ABSTRACT</u>	I
<u>DECLARATION</u>	II
<u>ACKNOWLEDGEMENTS</u>	III
<u>CHAPTER 1</u> An introduction to the early stages of ovarian follicle development.	1
<u>CHAPTER 2</u> Establishing the culture system.	39
<u>CHAPTER 3</u> A quantitative study of the development of isolated preantral follicles in collagen gel.	78
<u>CHAPTER 4</u> A morphological study of cultured preantral ovarian follicles after transplantation under the kidney capsule of ovariectomized hosts.	117
<u>CHAPTER 5</u> An autoradiographic investigation of hormonal effects on in vitro development of mouse ovarian follicles.	138
<u>CHAPTER 6</u> Preliminary experiments using a partially defined culture medium to explore the effects of epidermal growth factor on small follicular development.	175
<u>APPENDIX 1</u> Fixation and Staining	195
<u>APPENDIX 2</u> Published Material	197
<u>REFERENCES</u>	198

CHAPTER 1

An introduction to the early stages of ovarian follicle development.

INTRODUCTION

The mammalian ovary has two major functions: 1) the production of the steroid sex hormones and 2) the maturation and ovulation of the egg (oocyte). Both these processes take place within the follicle, the functional unit of the ovary, and depend on the continuous growth of follicles from primordial to mature Graafian stage. Depletion of the primordial follicle pool signals the end of the reproductive phase, marked by the menopause in the human female. The primordial follicle consists of an oocyte and a few pregranulosa cells while the Graafian follicle features three cell types - theca cells, granulosa cells and the oocyte. The growth of the follicle from primordial to Graafian stage is a complex process involving coordinated maturation of the oocyte and proliferation and differentiation of the granulosa and theca/stroma cells. The processes influencing follicle growth and atresia are of great interest. They are of interest to the reproductive physiologist and cell biologist and to the clinical scientist studying control of fertility or investigating causes of infertility. They are important in studying fertility and fecundity in domestic animals and endangered species. An additional area of interest is reproductive toxicology, toxicologists need to consider the harmful effects of drugs and environmental chemicals on the subtle processes controlling follicular growth and on the quality of the oocytes.

The investigation of the biology of the ovarian follicle requires the integration of techniques of many disciplines. Follicle development involves marked changes in structure and ultrastructure, changes in cellular synthesis and secretion, cell cooperation, changes in responsiveness to endocrinological signals and coordinated switching on and off of cell proliferation and the initiation of differentiation. Techniques from the

fields of light and electron microscopy, biochemistry and cell biology as well as the more classical physiological and endocrinological approaches are essential. This thesis is concerned with developing and utilising techniques which will permit the factors controlling recruitment and growth of small follicles to be investigated.

This introduction will review the biology of the ovarian follicle with particular reference to primordial and preantral follicles and identify areas requiring further research. Subsequent chapters will consider particular aspects of cell biology related to follicular development and the techniques developed to investigate them.

Origins and development of the mammalian ovary

The gonads originate as a ridge on the ventral cranial area of the mesonephros and rapidly thicken to form cylindrical bodies with rounded ends (Brambell, 1927; Byskov, 1986). Further remodelling in the embryonic ovary results in the rounded appearance of the ovary and the formation of the hilum and in some species the development of a bursa. The ovary also moves caudally to assume a lower abdominal or pelvic position. The stromal and epithelial cells of the ovary are of interest because in the adult they have remained able to differentiate into theca and granulosa cells during the process of folliculogenesis. The origin of these cells remains uncertain, coelomic epithelium, mesenchyme and mesonephric tissue may all contribute but the mesonephros probably makes the major contribution (see Byskov, 1986). However Byskov does not exclude a minor contribution from coelomic-derived epithelial cells. As current information is derived from studies using a morphological approach it remains difficult to be sure of the original lineage of somatic cells in the ovary. The pregranulosa cells are thought to be derived from the differentiation

of intraovarian rete cells and similarities between the rete cells and granulosa cells are marked (Byskov & Lintern-Moore, 1973). The germ cells of the ovary, the oocytes, arise from primordial germ cells which migrate into the sexually undifferentiated ovary from the yolk sac (at days 8-10 in the mouse embryo). The extragonadal origin of the primordial germ cells is now accepted for most species (Eddy et al., 1981) but some earlier theories will also be discussed later. PGCs which do not reach the ovary are usually lost (mouse - Snow & Monk, 1983; human - Jirasek, 1971) but some occasionally survive at extragonadal sites (mouse- Chiquoine, 1954; Upadhyay & Zamboni, 1982; man - Falin, 1969) where they may give rise to teratomas (reviewed by McIlhinney, 1983).

Ovarian morphogenesis has been summarised into three overlapping phases by Zamboni et al. (1980): colonization, organisation and compartmentalization. Colonization of the embryonic ovary by PGCs is followed by organisation as the ovary is invaded by connective tissue, blood vessels and the rete ovarii (mesonephros derived tubules). Compartmentalization follows as the ovarian connective tissue becomes denser and separates the inner and outer ovarian cell mass into the cortex and medulla. The inner cell mass contains the rete ovarii and forms the hilar and medullary areas of the ovary. The outer cell mass contains the developing germ cells and a scanty stroma and is the rudimentary cortex. Before moving on to a review of the main components of the adult ovary, which will include a more detailed description of the follicle at different stages of development, a brief consideration of the two main and interdependent developmental processes within the ovary, oogenesis and folliculogenesis, is presented.

Folliculogenesis and Oogenesis

As the embryonic ovary develops the germ cells undergo a number of mitotic divisions and are termed oogonia. At the cessation of mitotic proliferation the oogonia enter meiosis and are termed oocytes. The oocytes enter the prophase of the first meiotic division. In the mouse, and some other species, the initiation of meiosis may depend on the mesonephric tubules, possibly by secretion of a meiosis initiating substance. Byskov (1975) observed that in cat, mink and ferret, meiosis began close to the mesonephric tissue connecting to the ovary, the extraovarian rete, and Byskov et al. (1977) reported that when undifferentiated mouse ovaries were transplanted without the mesonephric tissue they did not enter meiosis. Byskov & Saxen (1976) demonstrated that co-culture of an undifferentiated mouse testis and an ovary with attached mesonephric tissue resulted in the induction of meiosis in the testis. O & Baker (1976) report similar results with fetal hamster gonads and Fajer et al. (1979) with newborn hamster ovaries. Other species such as the rabbit do not exhibit this effect (Byskov, 1979; 1986).

The oocytes are arrested at the late diplotene (or dictyate in rat - Beaumont & Mandl, 1962; and mouse - Slizynski, 1967) stage of prophase until meiosis is resumed. This period of arrested prophase may last in the human from birth to 50 years of age (Baker, 1971) and meiosis is only resumed shortly before ovulation (Edwards, 1966). At this stage the oocyte contains duplicate sets of chromosomes.

The diplotene oocyte becomes enclosed by pregranulosa cells which rest on a basement membrane or basal lamina: this marks the beginning of the process of folliculogenesis (Peters, 1978). The factors initiating folliculogenesis are unknown but if the oocyte is not incorporated into a follicle it generally degenerates (Ohno & Smith, 1964) although in some

species such as the cat unenclosed oocytes are common, especially in immature ovaries (Peters, 1978). Similarly an ovary without oocytes does not develop follicles. In Turner's syndrome, in the human, the few follicles present disappear early during development (Singh & Carr, 1966) and follicles are lost after irradiation in the mouse (Beaumont, 1961). The follicle at this stage is a primordial follicle and consists of pregranulosa cells and basal lamina. The follicles will remain in this quiescent state until recruited into the growth phase by the as yet unknown signal(s). Folliculogenesis and oogenesis are linked processes which usually occur together. Morphological changes and proliferation of the somatic cells of the follicle are the most dramatic changes in folliculogenesis and oogenesis is marked by expansion of the oocyte volume and formation of the zona pellucida.

When growth is initiated the pregranulosa cells differentiate and proliferate forming 1, 2, 3 layered and then multilaminar primary follicles. This increase in follicle size is accompanied by an increase in oocyte diameter and the oocyte almost reaches its maximum size in these preantral follicles. Stromal cells differentiate into theca and invest the primary follicle. With the formation of an antrum the multilaminar follicle becomes a secondary follicle. There is little additional increase in the oocyte diameter but the nuclear material tends to become more distinct and the nucleolus more compact. The nucleus with its distinct nucleolus is generally termed the germinal vesicle (Baca & Zamboni, 1967). As the secondary follicle grows the antrum increases in size and the theca organises into distinct theca interna and externa. The oocyte remains attached to one wall of the follicle by a mound of granulosa cells, the cumulus oophorus, developing around it. The mature antral follicle is also termed a Graafian follicle after its discovery in the 17th century by

Reiner de Graaf. The final, tertiary or preovulatory phase of follicular development is marked by the resumption of oocyte meiosis prior to ovulation. The onset of meiosis is heralded by the dissolution of the nuclear membrane - germinal vesicle breakdown. The oocyte moves into diakinesis, then metaphase, anaphase and telophase. Meiosis progresses and the primary oocyte divides unequally to form the secondary oocyte and the first polar body as a result of peripheral location of the spindle apparatus. Meiosis in vertebrates usually arrests in metaphase II of the second meiotic division. The secondary oocyte is ovulated and meiosis is usually completed only after fertilisation. The follicle shell left after ovulation undergoes further, extensive differentiation to form the corpus luteum. Ovulation is not, however, the fate of most follicles. The majority are lost through atresia during the process of folliculogenesis.

Although not a major focus of the present investigation, atresia is an important process in the ovary. Atresia refers to the processes of loss of follicle integrity and oocyte degeneration that results in the loss of the follicle other than by ovulation. At puberty the human ovary has some 400,000 oocytes present, some 400 of these are likely to be lost by ovulation, the rest are eliminated by the process of atresia (Block, 1952; Byskov, 1978a). Byskov (1978a) has estimated that 77% of follicles in the mouse ovary and 99% in the human ovary become atretic. Atresia is characterised by the cessation of granulosa cell mitosis, separation of the granulosa from the basal lamina and oocyte degeneration. Atresia may begin in any of the follicular compartments but the oocyte has been proposed as the site of initial changes. Follicles can become atretic at any stage but in a study of the cycling rat Hirshfield (1988) reported that most atretic follicles were in the early antral stage. However it is interesting to note the suggestion by Oakberg (1979) that in the intact cycling mouse follicles

may be committed to either atresia or normal development by the time three layers of granulosa cells have developed. Reviews of follicle atresia have been presented by Weir & Rowlands (1977), Byskov (1978a) and Centola (1983).

Studies in the mouse using [^3H]thymidine pulse labelling and autoradiography have demonstrated that the rate of follicle development is not constant over the life span. In the immature animal the rate of follicle loss is high and Pedersen (1969) has shown that more follicles enter the growth phase in the 7-day-old mouse than in the 3-5 week old animal. He also demonstrated faster follicle growth in the younger animal. It has been estimated that in the mature mouse a follicle takes some 17-19 days from initiation of growth to reach the Graafian stage (Hoage & Cameron, 1976; Pedersen, 1970a, b) but other authors using different methods estimate the rate of growth to be much slower, requiring more than six weeks (Oakberg, 1979). Only a small proportion of the growth period is spent in the antral phases of growth yet most research has focussed on follicle or granulosa cells from this stage.

The complexity of follicle dynamics and follicle selection can make it difficult to assess the impact and importance of treatments on follicle loss through follicle death or progression to the next stage and on the number of follicles available at each stage. In an attempt to analyse and describe follicle dynamics a mathematical approach using compartmental modelling has been developed by Faddy and co-workers (e.g. Faddy et al., 1976; 1983). Lacker and colleagues have used another approach to mathematical modelling to propose a theory of follicle selection during the ovarian cycle (Lacker et al., 1987a, b; Meuli et al., 1987).

Folliculogenesis represents a complex, sequential process of differentiation of at least three distinct cell types and the rest of this

review will try to illustrate some of the major morphological and functional changes which take place. Factors influencing follicular development (and atresia) will be considered as will the possible role of the major non-follicular tissues of the ovary. The major emphasis will be on the small follicle up to antral development.

Structure of the mammalian ovary and ovarian follicle.

In most mammals the ovary lies within a bursa, the bursa ovarica, which encloses a periovarian space (see Beck, 1972; Mossman & Duke, 1973). The bursa is absent in man and some other primates. In other species for example shrew and weasel it completely encloses the ovary (Eckstein & Zuckerman, 1956). The mouse, rat and dog have a complete bursa but the periovarian space is connected to the peritoneal cavity by narrow communicating passage (Wimsatt & Waldo, 1945; Alden, 1942; Kellogg, 1941). The ovary is covered by a mesothelium and contains follicles of varying stages, stromal and connective tissue, blood and lymphatic vessels and nerves. In some species the ovary may also contain luteal and interstitial tissue.

The surface epithelium.

The ovarian surface is covered by a serous membrane continuous with the peritoneum - the germinal or surface epithelium. The surface may be smooth but invaginations and folds are seen in some species e.g. rabbit (Motta, 1974) and dog (Andersen & Simpson, 1973). The surface epithelium of the rabbit ovary consists of a single layer of cuboidal, usually ciliated, cells interspaced with occasional flatter cells resting on a distinct basement membrane and the outer aspect of the cuboidal cells is convex giving a scalloped appearance to the ovarian surface (Björnsing & Cajander, 1974a, b). Weakly (1967) describes a similar mix of cuboidal and flat cells

in the hamster surface epithelium but does not mention cilia. Germ cells have been identified in fetal surface epithelium (e.g. human - Motta & Makabe, 1982) but Zamboni et al.(1980) states that the germs cells remain in the stroma and even the most superficial are separated from the surface epithelium by a basal lamina. Waldeyer (1887), cited in Baker (1972), suggested that germ cells originated through proliferation of the surface epithelium during embryogenesis but subsequently revised this opinion. As discussed above an extragonadal origin has been demonstrated (Everett, 1943; Nieuwkoop, 1949).

The contribution of the surface epithelium to the somatic cells of the follicle is uncertain. Many authors argue against any contribution from the surface epithelium to the cells of the follicle unit (e.g. Wischnitzer, 1965; Zamboni et al., 1980). A light and electron microscope study of the hamster ovary from 24 hours *pre-partum* to 8 days *post-partum* contained no evidence suggesting any contribution of the surface epithelium to the follicular unit (Weakly, 1967) and histochemical studies of the canine ovary support this conclusion (O'Shea, 1966). Byskov et al (1977) investigated the influence of the surface epithelium and rete ovarii on follicle formation in the mouse. Removal of the rete ovarii prevents follicle formation, absence of a surface epithelium had no effect on folliculogenesis. However Motta & Makabe (1982), from EM studies of human fetal ovaries, suggest that both the sex cords (columns of cells derived from proliferation and inward migration of the mesonephros and coelomic epithelium) and surface epithelium may contribute somatic cells to the follicle. In some species marked proliferation of the surface epithelium occurs after folliculogenesis is under way (e.g. human - Gondos, 1975; dog - Andersen & Simpson, 1973; rabbit - Gondos, 1969). In the mouse, and possibly other species, the surface epithelium may contribute to folliculogenesis but is

not essential for the formation of follicles.

The role of the surface epithelium in the mature ovary is uncertain. The epithelium exhibits morphological changes in response to hormones and during ovulation (Björnsing & Cajander, 1974a, b). In the mare the surface epithelium is modified and only covers a groove, the ovulation fossa, where follicles rupture at ovulation ((O'Shea, 1968). Björnsing & Cajander (1974a, b) have shown that the surface epithelial cells increase in size, lose microvilli and develop dense round cytoplasmic bodies 4 hours after injection of HCG. Approximately 2 hours before ovulation the granular content of these bodies, thought to be lysosomal enzymes, decreases (Cajander & Björnsing, 1975). The work of Cajander and Björnsing (Cajander & Björnsing, 1975; 1976; Cajander, 1976) has resulted in the hypothesis that the surface epithelium lysoenzyme contributes to the proteolytic disintegration of the follicle wall prior to follicle rupture. This view has been disputed by the observations of Rawson & Espey (1977). They report that both the number and size of EM-dense granules in the cytoplasm of rabbit surface epithelial cells increases up to the point of ovulation with a decrease observed only after ovulation has occurred. They suggest that the granules are not lysosomes (see Espey, 1980) and that the pre-ovulatory increase in these granules is not a cause of ovulation. Currently a specific protease, plasminogen activator, is receiving much attention and Lipner (1988) gives a recent review of this and other aspects of mammalian ovulation.

The primordial follicle

The primordial follicle consists of an oocyte surrounded by a single layer of flattened epithelial pregranulosa cells probably derived from the rete ovarii (Byskov & Lintern-Moore, 1973). The size of the primordial

follicle varies greatly between species, from as small as 15 μm diameter in the mouse (Gosden & Telfer, 1987) to 50 μm -70 μm diameter in the human (Baca & Zamboni, 1967; Nicosia, 1983). In many species the primordial follicles form a peripheral zone close to the surface epithelium although they can be more widely distributed. Primordial follicles retain the same appearance and size despite advancing age: a primordial follicle is essentially the same in a child as in a premenopausal women (Peters, 1978). Occasionally the primordial follicle may contain more than one oocyte and polyovular follicles have been noted in man, monkey, dog, cat, ferret, pig, sheep, goat, rabbit, mouse, rat, guinea pig, prairie dog, bat armadillo, kangaroo, native cat and opossum (see Brambell, 1956) and several other species. The incidence of these structures varies considerably between species and they are more common in fetal and immature ovaries. Polyovular follicles are also seen at later stages of growth (Mossman & Duke, 1973, Telfer & Gosden, 1987).

The number of pregranulosa cells of the mouse primordial follicle have been reported as 2-8 cells by Lintern-Moore & Moore (1979) and between 2-8 cells by Telfer et al. (1988). Telfer and co-workers reported that most follicles had between 3-6 pregranulosa cells and by using an assay for an X-linked alloenzyme PGK-1 (phosphoglycerate kinase-1) estimated that all the granulosa cells of antral follicle were derived from about five cells. The pregranulosa cells are elongated and in places the follicle wall can be as thin as 0.5 μm (Hope, 1965). The pregranulosa cells do not form a continuous layer round the oocyte and the gaps in the follicular wall may provide a direct route for transport of extracellular substances to the oocyte (Zamboni, 1974; 1976; 1980). The pregranulosa cells are relatively simple, undifferentiated cells with an elongated nucleus. The nuclear envelope is highly irregular due to many folds and indentations (Zamboni,

1974; 1976; 1980, Cran et al., 1979). Cytoplasmic structures include numerous mitochondria, a small Golgi complex, a few randomly distributed cisternae of mainly granular endoplasmic reticulum, lipid droplets, and free ribosomes (Dvorak & Tersarik, 1980). At this point they do not have the appearance of steroid producing cells typified by mitochondrial morphology, abundant ER and lipid inclusions.

The pregranulosa cells appear to have a supportive (possibly physical and metabolic) function since naked oocytes generally degenerate (Ohno & Smith, 1964) but direct metabolic coupling has not been demonstrated. Gap junctions between the oocyte and pregranulosa cells have been demonstrated (Anderson & Albertini, 1976; Mitchell et al., 1986). It also appears that contact with the pregranulosa may have a role in initiating growth (see below).

The epithelial layer is separated from surrounding stromal tissue by a thin, granular basal lamina (Anderson et al., 1978). The zona pellucida is not yet obvious but Guraya (1970a; 1974a) has reported PAS-positive aggregations at the epithelial cell-oocyte junction of the human primordial follicle that represent the beginning of the zona pellucida. Material resembling zona pellucida has also been reported in primordial sheep follicles (Cran et al., 1979). However in the mouse the zona is not secreted nor is there gene transcription for zona proteins until after the initiation of follicle growth (Wasserman, 1988). The follicles are surrounded by a dense connective tissue containing spindle-shaped cells usually designated as fibroblasts, smooth muscle cells, blood vessels and bundles of collagen and reticular fibres (Zamboni, 1972; 1974; Osvaldo-Decima, 1970).

The primordial oocyte.

The diplotene primordial oocyte has a relatively simple structure with a spherical nucleus (about 22-24 μm diameter in the human - Hertig, 1968) often eccentrically located (Zamboni, 1972; 1974). The nucleus contains one or more granular nucleoli, some 2-3 μm in diameter (Baca & Zamboni, 1967; Dvorak & Tesarik, 1980). The cytoplasm contains an irregular distribution of structures including mitochondria, lipid droplets and vacuoles but in some species many of the cytoplasmic organelles are grouped together near one pole of the nucleus forming the paranuclear complex or Balbiani's vitelline body (Hertig, 1968; Dvorak & Tesarik, 1980). This body includes the Golgi complex, mitochondria and lipid inclusions and the centrally located cytocentrum surrounded by a dense endoplasmic reticulum (see Ullmann, 1978; Guraya, 1985 for more complete descriptions).

Although the primordial oocyte is sometimes described as quiescent, with the dictyate state referred to as a resting phase (Dvorak & Tesarik, 1980, Baker, 1972) the oocyte is metabolically active with slow, but ongoing, protein and RNA synthesis (Baker, et al., 1969; Baker, 1971; Lintern-Moore & Moore, 1979; Oakberg, 1967; 1968). Schultz et al. (1979) using [^{35}S]methionine estimated the rate of protein synthesis in primordial oocytes as 1.1 pg/h (compared with 45 pg/h in a mature oocyte). However the mature oocyte has many times the volume of the primordial oocyte. As described, gaps in the pregranulosa layer may provide direct access of nutrients to the oocyte and Anderson (1972) has demonstrated that intravenously injected horseradish peroxidase can move through these gaps to the oocyte. However metabolic cooperation with surrounding pregranulosa cells is also probable (Zamboni, 1976; Guraya, 1985). The membranes of the oocyte and pregranulosa cell lie in close apposition. In some areas the membranes are joined by desmosomes, in others there are

areas of lacunae featuring interdigitating microvilli (Zamboni, 1974). Pinocytic vesicles are seen in both plasma membranes (Guraya, 1977).

Initiation of follicle growth

In most species the pool of primordial follicles is established during fetal life and subsequent ovarian development is marked by the progressive depletion of this finite pool as follicles are recruited into the growth phase to become primary follicles (Jones & Krohn, 1961). Once growth has been initiated the follicle is irreversibly committed to progressive development towards ovulation or atresia. Recruitment from the primordial pool into the growth phase is a continuous process. Movement from the primordial pool continues uninterrupted regardless of the endocrine status of the animal (Peters et al., 1975). A few primordial follicles enter the growth phase daily even during major endocrine events such as pregnancy or lactation. In some, larger species follicle recruitment commences during fetal life (e.g. cow - Mauleon, 1967; human and monkey - van Wagenen & Simpson, 1965) and in other, smaller species growth is initiated in the perinatal period (mouse - Brambell, 1927; rat - Beaumont & Mandl, 1962; hamster - Challoner, 1974). The postnatal initiation of folliculogenesis in the mouse makes it a useful model for studying the kinetics of follicle development (Lunenfeld et al., 1975).

The nature of the signals initiating the transition from primordial to growing follicle is unknown but intraovarian factors arising from the oocyte (Baker, 1972) or pregranulosa cells (Lintern-Moore & Moore, 1979) rather than systemic factors may be important for regulating recruitment. The size of the primordial pool may be one factor (Krarup et al., 1969). Loss of primordial follicles, perhaps due to loss through the surface epithelium during the first postnatal week (Byskov & Ramussen, 1973)

reduces the size of the pool as the animal matures and results in a marked reduction in the number of primordial follicles entering the growth phase. Artificial reduction of the primordial pool by injection of dimethylbenzanthracene produces the same effect (Krarup, et al., 1969). Another factor suggested by Peters et al (1973) is the degeneration of larger follicles with release of a substance inhibiting follicle initiation but this remains unconfirmed. Atresia of larger follicles is more prominent in the mature animal.

The role of the gonadotrophins, LH (luteinizing hormone) and FSH (follicle stimulating hormone) in initiation of growth is controversial. A 'priming' action of the gonadotrophins appears to be essential for follicle growth to begin in human fetuses and in neonatal mice. Baker & Scrimgeour (1980) compared the ovaries of normal and anencephalic human fetuses (low in circulating gonadotrophins). They reported proliferation of the oogonia and formation of primordial follicles in the affected fetuses but an absence of growing follicles beyond the primordial stage as found in normal fetus controls. Injection of antiserum to LH and FSH in neonatal mice from the day of birth similarly blocked the development of growing follicles (Eshkol et al., 1971). Lintern-Moore (1977) has demonstrated that exogenous gonadotrophin (PMSG) could increase the number of growing follicles if given from birth to day 5, suggesting there may be a critical period when the gonadotrophins can influence the initiation of growth.

However the gonadotrophins do not appear to be obligatory for further small follicle development in some species. Hypophysectomy of mature rats and mice does not disrupt primordial follicle recruitment (Ingram, 1953; Jones & Krohn, 1961; Nakano et al., 1975; Faddy et al., 1983). Mulheron et al (1987, 1989) used antiserum to rat FSH and LH to investigate the distribution of these hormones in the rat preovulatory follicle. They

detected gonadotrophins in some primordial and preantral follicles and investigated this distribution during the oestrous cycle. In most of the primordial follicles gonadotrophins were not detected but FSH was identified in the cytoplasm and germinal vesicle of the oocyte and LH was present in both pregranulosa and oocyte compartments of reactive primordial follicles. The authors report an increased number of hormone-positive primordial follicles on the day of proestrus. The presence of the gonadotrophins in some primordial follicles suggests they could have a role in growth initiation.

In a study of recruitment in normal and dwarf Snell mice (which have low levels of gonadotrophins) an effect on small follicle growth was noted but the role of FSH as the trigger for primordial recruitment was not supported (de Reviers, 1988). Further evidence for some role in primordial recruitment comes from modelling studies on hypogonadal mice (Halpin et al 1986).

Hirshfield (1989) has used long-term continuous infusion of tritiated thymidine to investigate growth in the very small follicle of cyclic adult rats. She concluded that a substantial fraction of small follicles in the nongrowing pool were in fact growing but at a slow rate, taking more than a month and 3-4 mitotic cycles to grow from 3-4 to more than 20 granulosa cells. Hirshfield suggests that pulse labelling with [³H]thymidine does not allow sufficient time for the slowly proliferating granulosa cells to enter the S-phase and incorporate the radiolabelled thymidine. Lintern-Moore (1977) has assumed that the transition from squamous to cuboidal shape represents the beginning of growth in the granulosa cell and other authors have considered this transition essential for granulosa proliferation (Franchi & Baker, 1973). Hirshfield (1989) reported labelling of both squamous and cuboidal granulosa cells of the smallest follicles.

The preantral growing follicle.

The preantral follicle initially consists of a growing oocyte and proliferating membrana granulosa. Later a thecal layer develops. The preantral stage of development extends from recruitment from the primordial pool to the initiation of antral development. The first indications of follicle growth are mitotic activity within the epithelial cells and the development of regions of zona pellucida material (Zamboni, 1974). The pregranulosa cells begin to proliferate and form a single, continuous layer around the oocyte. The cells become more cuboidal or columnar in shape forming a distinctive unilaminar follicle.

Continued proliferation of the membrana granulosa results in additional layers forming around the centrally located oocyte. In 1,2 and 3 layered follicles the granulosa maintains an orderly distinct arrangement of the layers. As the follicles become multilaminar the cells may lose this orderly arrangement of distinct layers and in large multilaminar follicles lacunae appear. The granulosa cells remain cuboidal/polyhedral but with a layer of columnar granulosa cells resting on the basal lamina and form a substantial investment around the oocyte (see Zamboni, 1974; Guraya, 1985). In the mouse the granulosa cells will undergo 11-12 mitotic divisions forming up to 10 layers in a solid follicle of some 2000-3000 cells and diameter of 150-400 μm before antral formation is incipient (Gosden & Telfer, 1987; Gosden et al., 1988).

Compared with the pregranulosa cell the nucleus of the granulosa cell enlarges and becomes irregular in shape. It develops marked indentations and may assume a lobulated appearance with two or more lobes connected by a thin chromatin bridge (Zamboni, 1974; Guraya, 1977; Bjersing, 1978). At this stage the granulosa cell nuclei are reminiscent of the nuclei of homologues in the testis, the Sertoli cell (Dym, 1973). A large distinct

nucleolus is a feature of the granulosa cell at this stage. Cytoplasmic organelles do not differ in individual appearance from those in the primordial follicle (Dvorak & Tesarik, 1980). Stankova et al (1985) report an increase in the volume fraction represented by the Golgi apparatus and a decrease in secondary lysosomes in unilaminar preantral follicles compared with primordial follicles. There is some evidence that the granulosa cells in the large preantral follicle are not homogeneous (Kasson et al., 1985), the topic of granulosa cell heterogeneity will be developed in the next section.

Theca cells

The initial stages of small follicle growth involve only the oocyte and granulosa cells and the follicle is surrounded by an undifferentiated stroma. Fibroblast-like stroma cells begin to align themselves concentrically around the multilaminar follicle. In the mouse a theca is usually distinct by the time 3-4 layers of granulosa cells are apparent (Oakberg, 1979). In the rat some stromal alignment is seen 7 days after birth when the follicle has 4 layers of stratified granulosa cells and by day 9 a concentrically arranged theca is seen. By day 12 when the antrum becomes evident the theca is distinct (Carson & Smith, 1986). The mesenchymal origin of thecal cells is indicated by the work of Huira & Fujita (1977), and Hoage & Cameron (1976). The factors initiating and controlling this process are largely unknown although Dubreuil (1957) has suggested induction by the granulosa cells. The large nucleus can be irregular or elongated and cytoplasmic organelles include a granular ER, free ribosomes, a small Golgi complex and a few small mitochondria with transverse cristae: they show none of the subcellular indicators of steroidogenesis (see Dvorak & Tesarik, 1980; Guraya, 1985). The thecal cells are separated from the

granulosa layers by a basal lamina which develops to 1-2 μm thick (Dvorak & Tesarik, 1980) and has been described in the mouse by Anderson et al (1978) as a bipartite structure composed of an homogenous inner stratum and an outer region of collagen-like fibres.

The primary oocyte

The initiation of granulosa cell proliferation is accompanied by growth of the oocyte. Granulosa and oocyte both exhibit an increase in size and complexity (Zamboni, 1980; Dvorak & Tesarik, 1980). Kaur & Guraya (1983) demonstrated a linear relationship between oocyte and follicle diameter in the early stages of follicle growth in the mole rat. The oocyte reached its maximum diameter of 60 μm at a follicle diameter of about 120 μm : follicle diameter continued to increase to about 600 μm . Green & Zuckerman (1951) demonstrated a broadly similar pattern in the human ovary, with the maximum oocyte diameter about 80 μm with a follicle diameter of 110 μm . Brambell (1928) reported a similar pattern in the mouse with a maximum oocyte diameter of about 70 μm in follicles of 125 μm . Lintern-Moore & Moore (1979) reported an increase in mouse oocyte RNA polymerase activity when the follicle had 9 granulosa cells in the widest cross-section and a detectable increase in oocyte area with 10 follicle cells. This is the first biochemical indication of growth initiation.

The nucleus enlarges and cytoplasmic changes occur, Balbiani's vitelline body moves away from its paranuclear position and disappears as its components are distributed within the ooplasm, mainly aggregated in the cortical ooplasm leaving the central areas of the oocyte relatively clear (Zamboni, 1972; Guraya, 1974). The Golgi complex also disperses into aggregates in the peripheral ooplasm and cortical granules (which play a role in the block to polyspermy) develop at this stage (Baca & Zamboni,

1967). Stankova et al (1985) report an increase in the volume fraction occupied by mitochondria, granular ER, and Golgi apparatus in the preantral oocyte and Zamboni (1972) also reported the increase in granular ER.

Zona pellucida

Wolgemuth et al. (1984) used antibodies to zona pellucida proteins to investigate zona formation in the rabbit. The appearance of zona material in the cytoplasm of primordial, primary and secondary oocytes is reported. The authors concluded that extracellular deposition of zona pellucida material occurs before the epithelial cells become cuboidal. Leveille et al. (1987) using an immunofluorescence technique demonstrated the presence of zona material in the ooplasm but not granulosa cytoplasm in hamster follicles entering the growth phase and *in vitro* studies indicated that the oocyte was the main source of zona proteins (e.g. Bleil & Wasserman; 1980, Shimizu et al., 1983). Focal deposition of zona material begins to separate the oocyte and granulosa cells. Formation of a continuous zona is usually complete in the bilayered follicle (Wolgemuth et al., 1984). The genes involved in the formation of the zona pellucida are not expressed until oocyte growth has been initiated (Philpott et al., 1987; Wasserman, 1988a).

The oocyte is now surrounded and isolated from the vascular system by two barriers: the zona pellucida and the continuous layer(s) of granulosa cells and it is dependent on metabolic cooperation with the surrounding granulosa cells. Development of the zona pellucida is accompanied by the development of numerous cellular processes between the oocyte and granulosa cells. The granulosa cells extrude thin cytoplasmic processes that traverse the zona pellucida to attach to the oocyte surface. The oocyte develops numerous microvilli and micropapillae which are well developed

around the regions next to granulosa cell processes (Zamboni, 1972; 1974; Guraya, 1974; Albertini & Anderson, 1974; Anderson & Albertini, 1976; Dvorak & Tesarik, 1980, Tesoriero 1981). The occurrence of gap junctions and desmosomes between granulosa cells and oocytes has been demonstrated by EM studies in the mouse, rat and rabbit (Anderson et al., 1978). Anderson & Albertini (1976) also reported gap junctions between the granulosa cells in the preantral follicle.

Steroidogenesis and the preantral follicle

In general the ovary is unable to produce steroids before the formation of the follicle although in certain species some of the enzymes involved in steroidogenesis have been identified before the onset of folliculogenesis (e.g. mouse - Hoyer & Byskov, 1981; rabbit - Milewich et al., 1977; human - George & Wilson, 1978). The first, minimal, expression of steroidogenic activity probably occurs in the preantral follicle but the full steroidogenic potential is reached in the antral follicle. Evidence for a limited steroidogenesis in preantral follicles is mainly indirect due to the low levels of steroids present and the difficulties involved in isolation of intact preantral follicles (Gore-Langton & Armstrong, 1988). Granulosa cell receptors for FSH have been identified in the early preantral follicle of the rat (Presl et al., 1974, Nimrod et al., 1976) and hamster (Roy et al., 1987). Larger preantral follicles with theca also have receptors for LH (hamster - Oxberry & Greenwald, 1982; Roy et al., 1987). Reddoch & Armstrong (1984) demonstrated the production of oestradiol, progesterone and androstenedione by 6-day old (but not younger) rat ovaries *in vitro*. In this study large preantral follicles were the most mature follicles present.

Granulosa cells, from immature rat ovaries, are able to convert

pregnenolone to progesterone and 20 α -dihydroprogesterone in culture and dispersed ovarian cell cultures are able to convert testosterone to 5 α -androstane-3 α ,17 β -diol and androsterone (Goldring & Orly, 1985; Dorrington & Armstrong, 1979). However these effects take about 24 hours to develop and are in response to FSH. Histochemical evidence suggests that the enzyme Δ^5 -3 β -hydroxysteroid dehydrogenase is only weakly expressed in the preantral follicle but activity is abundant in the preovulatory follicle (Hoyer & Anderson, 1977). Enzyme induction is stimulated by FSH (Dorrington & Armstrong, 1979). Carson & Smith (1985) investigated steroidogenic activity in the rat ovary over the first 20 days of life. They concluded that ovarian aromatase activity increased significantly on day 7 but that due to low levels of progesterone and testosterone actual production of oestrogen was limited until day 12. Aromatase activity on day 7 correlated with the appearance of a distinct membrana granulosa and androgen production with the differentiation of theca on day 12. Terranova & Garza (1983) isolated preantral follicles from cyclic hamsters and studied steroid production after incubation *in vitro*. Follicles isolated before the LH surge produced mainly androstenedione: after the surge only progesterone was produced. From the literature it is clear that the preantral follicle cells can respond to gonadotrophic stimulation with induction of steroidogenesis although there is at present no clear evidence that this response occurs *in vivo* (see Gore-Langton & Armstrong, 1988).

Factors influencing development of the preantral follicle

A number of factors may influence the development of the preantral follicle and these include systemic factors such as the gonadotrophins and intraovarian factors such as follicle number, the ovarian steroids and cell-

cell interactions. The role of intraovarian factors in folliculogenesis is of current interest. The important role granulosa cells play in oocyte growth is well recognised (e.g. Eppig, 1977; Herlands & Schultz, 1984; Buccione et al., 1987) but the possibility of oocyte regulation of granulosa cell differentiation and function has not been extensively explored. Some evidence from cumulus oophorus cultures indicates that the oocyte does secrete factors which influence granulosa cell differentiation (Buccione et al., 1990; Vanderhyden et al., 1990). The role of growth factors as paracrine regulators of follicular growth is considered later (chapter 6).

As discussed above, the gonadotrophins are not obligatory for small follicle development but a role in modulating growth in the preantral follicle is likely (Arendsen de Wolff-Exalto, 1982; Roy & Greenwald, 1986; 1989). In immature, hypophysectomized or hypogonadal mice studies of follicle dynamics have all reported increased rates of preantral follicle growth when compared to normal mice (Faddy et al., 1976; 1983; 1987; Halpin et al., 1986). Receptors for FSH are found on the granulosa cells and receptors for LH on the theca. In the mouse there is no detectable variation in the numbers of preantral follicles during the oestrous cycle (Pedersen, 1970a). In gonadotrophin-deficient dwarf Snell mice injection of FSH has been shown to increase the labelling (measured using [³H]thymidine) to approximately the same level as in the normal controls (de Reviere, 1988).

However in the rat the number of small follicles does vary during the oestrous cycle (Butcher & Kirkpatrick-Keller, 1984). Changes in small follicle numbers are not seen during the hamster cycle but an increase in preantral follicle incorporation of [³H]thymidine on proestrous and oestrous days has been demonstrated (Chiras & Greenwald, 1980). Dhanasekaran & Moudgal (1986) suggest that for preantral follicles in the

rat a lack of FSH is the essential signal inducing atresia. Greenwald & Terranova (1988) have reviewed the effect of hypophysectomy in several species and identified a major quantitative disruption of small follicle growth although some follicles may continue to grow. Hypophysectomy also alters the secretion of other hormones such as growth hormone and thyroxine which may alter ovarian function (Howe et al., 1978; Halpin et al., 1986).

Ovarian steroids are major candidates as intraovarian or paracrine regulators of follicle growth and function but much of the current work relates to antral and preovulatory follicles (see reviews by Hillier, 1985; 1987) and direct *in vivo* effects on preantral follicles can be difficult to establish. The effect of steroids and other hormones and growth factors on granulosa cell growth has been studied *in vitro* using cells obtained from large, preantral follicles of oestradiol or diethylstilboestrol treated, hypophysectomized immature rats. This experimental model enables the harvesting of large numbers of "relatively" undifferentiated preantral, granulosa cells (Hsueh et al., 1983, see also Erickson, 1983). Membrane receptors for FSH and intracellular steroid receptors, including those for oestrogen, progesterone and androgen, have been identified (Richards, 1979; Schreiber & Ross, 1976; Schreiber & Hsueh, 1979).

Oestrogen stimulation of the hypophysectomized rat stimulates proliferation of the membrana granulosa producing multiple layers of granulosa cells and increases cell responsiveness to FSH (Hsueh et al., 1983). Merk et al. (1972) reported that oestrogen administration increased the number of gap junctions in the membrana granulosa of hypophysectomized rats. When cultured without gonadotrophin the cells remain undifferentiated and only weakly steroidogenic but in the presence of FSH they acquire the features of granulosa cell cytodifferentiation

including LH receptors and increased steroidogenesis (Erickson, 1983). This system is a useful model for granulosa-luteal differentiation and results obtained are probably more representative of mature Graafian rather than preantral follicles.

Gondos (1982) used Silastic implants of oestradiol, dihydrotestosterone (DHT) or combinations of both steroids to investigate atresia of preantral follicles of the hypophysectomized immature rat. He reported that oestradiol alone or in combination with DHT reduced the percentage of atretic follicles compared with saline implant controls. Species differences are certainly important: Kim et al. (1984) reported a stimulatory effect of oestrogens on large preantral follicles in the hypophysectomized rat but not in the hamster.

During pregnancy the ovary is anovulatory; in some species Graafian follicles fail to develop and in others they may form but are unable to mature and become atretic (Greenwald, 1966; Pedersen & Peters, 1971). In the pregnant hamster large healthy antral follicles develop and ovulation can be stimulated by human chorionic gonadotrophin (Greenwald, 1967). Progesterone appears to block large follicle development but its effects on small and preantral follicles has received less attention. Pedersen & Peters (1971) investigated follicle dynamics in pregnant mice using tritiated thymidine labelling of mitotic cells. The results showed little difference in follicular growth rate between pregnant and nonpregnant animals but did indicate reduced recruitment from the non-growing pool. In contrast Telfer et al. (1990) used progesterone implants to suppress ovulation in young, adult mice and found that the results indicated that all stages of follicle growth were affected. Results of differential follicle counts were analysed using a compartmental model and demonstrated that the number and rate of growth of preantral follicles. However some follicles failed to develop an

antrum and their development was prematurely terminated by atresia. Since progesterone is known to reduce the pulsatile release of gonadotrophins it is unlikely that changes in hormone levels are responsible for the accelerated growth rates, though they probably account for the increased atresia. The authors speculate that in the absence of large follicles an endogenous inhibitor of follicle recruitment is deficient.

Androgens appear to have an inhibitory effect on follicular development and to promote atresia (Payne et al., 1956; Louvet et al., 1975a; Zeleznik et al., 1979; Hillier & Ross, 1979). Payne et al. (1956) demonstrated that testosterone propionate administration exerted a dose-related inhibition of ovarian weight gain and preantral follicle development in diethylstilboestrol stimulated hypophysectomized immature rats. A dose-related reduction in ovarian weight and increased follicular atresia in response to hCG were reported using the same animal model by Louvet et al. (1975b). Louvet et al. (1975a) used chemical and biological antagonists to androgen to reverse the effects of hCG, suggesting the involvement of androgens in the response to the gonadotrophin. Hillier & Ross (1979) also utilised the oestrogen-primed immature rat model showing that testosterone decreased ovarian weight, stimulated follicle atresia but had no effect on intraovarian progesterone levels. Gondos (1982) reported that DHT increased the percentage of atretic follicles of the hypophysectomized immature rat and that this effect was opposed when DHT was given in combination with oestradiol. Interestingly ultrastructural studies indicate that the initial changes in atresia occur in the granulosa cells rather than the oocyte (Gondos, 1982, Hay et al., 1976).

Other factors with potential effects on preantral growth include theca-granulosa cell interactions, other hormones (e.g. prolactin, inhibin, insulin), growth factors (e.g. epidermal growth factor, transforming growth

factor, insulin-like growth factors, fibroblast growth factor), and interactions with extracellular matrix. However, as most of these factors have been investigated using cells from antral follicles they will be considered in the next section and in more detail in subsequent chapters. The zona pellucida may also have an influence on follicle development. Rabbits immunized with porcine zona pellucida demonstrated disrupted follicular growth (Skinner et al., 1984). Within 7 weeks of immunization the number of follicles of all sizes was reduced and by 23 weeks very few growing follicles were seen. This study is the only evidence of a preovulatory role of the zona pellucida.

The antral follicle

As the main focus of this work is on the preantral follicle a detailed review of the antral follicle will not be attempted but main areas of follicle differentiation which may have implications for control mechanisms in the early stages of growth will be considered because these will include antrum formation, early antral granulosa and theca development and differentiation. Steroidogenesis and the role of follicular fluid are two major aspects of antral follicle function but the extensive literature covering these topics is not reviewed here.

Antrum formation marks the beginning of a period of rapid follicular growth although oocyte volume has reached its maximum. The physiological factors underlying the formation of follicular fluid have been recently reviewed by Gosden et al. (1988). Qvist et al. (1990) have demonstrated antrum formation *in vitro* suggesting that culture techniques will enable more extensive investigation of the mechanisms of follicular fluid formation to proceed. Gosden & Telfer (1987) examined data on the scaling of ovarian follicles from 22 mammalian species. In all the species studied

follicles had reached a mean diameter of 0.3 mm when they began to accumulate follicular fluid. In the mouse the granulosa cells can undergo 11-12 mitotic cycles resulting in a follicle of 2000-3000 cells, with a diameter between 150-400 μ m, before antrum formation is initiated (Gosden et al., 1988). Granulosa cells are separated by irregular lacunae and follicular fluid initially pools in these spaces. The pools of fluid coalesce to form a single antrum. The antrum is an almost spherical, centrally located cavity surrounded by a uniform volume of granulosa cells apart from at the pole where the cumulus cells investing the oocyte are attached. As follicle growth progresses the antrum increases in size and the thickness of the mural granulosa cell layers becomes attenuated.

In some species small vesicular structures, Call-Exner bodies, are found in the membrana granulosa of antral and large preantral follicles. Gosden et al. (1989) identified Call-Exner bodies in 5 out of 19 mammalian species investigated. These were human, rhesus monkey, rabbit, guinea-pig and sheep. Motta & Nesci (1969) add goat and possibly pig to the list of representatives. In the rabbit Call-Exner bodies ranged in size from 0.2 μ m to 30 μ m diameter (Gosden et al., 1989). These bodies appear to be in communication with the antrum and to contain follicular fluid. Their significance is uncertain but some sort of secretory function seems probable. Call-Exner bodies have been proposed as sites of active secretion of follicular fluid (Brambell, 1956) but as they remain distinct from the initial pools of follicular fluid formed and do not expand with the antrum any major contribution to antrum formation is unlikely. Additionally Call-Exner bodies are found in human granulosa cell tumours where they are a useful diagnostic feature occurring independently of an antrum (Novak & Woodruff 1967).

The signal initiating antrum formation is unknown but some of the

general requirements have been elucidated. Gonadotrophins appear to be essential since antrum formation is blocked in hypophysectomy and Qvist et al., (1990) identified FSH as an essential component for antrum formation *in vitro*. Antrum formation also marks the differentiation of the theca into a theca interna and externa and the acquisition of the follicles true steroidogenic capacity. Also it is at this stage that in some species the oocytes acquire the ability to resume meiosis when isolated from the granulosa cells.

With antrum formation two populations of granulosa cells are established - the granulosa cells forming the follicle wall, the mural granulosa or membrana granulosa, and the granulosa cells investing the oocyte, the cumulus oophorus (Mossman & Duke, 1973; Zamboni, 1974; 1976; Cran et al., 1979). In small and medium sized antral follicles the mural and cumulus granulosa cells of normal follicles appear to be very similar: cells nearest the basement membrane or zona pellucida cells are columnar and the remaining cells more rounded or polyhedral with larger irregular spaces between the cells. At this stage there is little difference between the two cell populations at the light or electron microscope level: the nucleus is spherical or oval with prominent reticular nucleoli. Many of the cells are mitotic and binucleate cells may be seen (Guraya, 1985; Dvorak & Tesarik, 1980).

As the antrum enlarges the two populations become more distinct, with structural and functional differentiation. The mural layers attenuate and the granulosa cells cease to proliferate, mitosis being restricted to the granulosa cells in the cumulus oophorus (Gosden et al., 1983). The mitotic rate in the cumulus cells results in its continued growth and these cells also develop lacunae, some of which communicate with the antrum. The cumulus cells do not demonstrate any polarity. The oocyte initially is

embedded in one side of the follicle with the cumulus cells forming a mound over it. As the antrum increases in size the cumulus oophorus and associated oocyte are displaced into the antrum but remain attached to the follicle wall by a bridge of cumulus cells. The inner layers of cells surrounding the oocyte become more columnar in shape forming the corona radiata. Even within these two populations further diversity is possible, mural granulosa cells lining the antrum appearing to be distinct from those adjoining the basal lamina.

These changes are associated with cessation of mitosis and FSH-induced acquisition of LH receptors (see Amsterdam & Rotmensch, 1987). Mural and cumulus granulosa cells may have distinct functions. LH binding studies indicate that mural cells near the basal lamina have more LH receptors than antral cells or cumulus cells (Amsterdam et al., 1975) and they are more actively steroidogenic, containing higher levels of 3β -hydroxysteroid dehydrogenase and cytochrome P-450 activity (Zoller & Weisz, 1978; 1979; 1980). Prolactin receptors show the reverse pattern, they are more abundant in the antral than mural granulosa (Dunaif et al., 1982). Gilula et al. (1978) have demonstrated that mural but not cumulus cells secrete plasminogen activator. In some species the mural cells secrete glycosaminoglycans while the cumulus cells secrete hyaluronic acid during the process of mucification (Yanagishita & Hascall, 1979). Clearly the granulosa component of the follicle demonstrates a definite heterogeneity with subpopulations exhibiting distinct structural and functional differences (Kasson et al., 1985; Erickson et al., 1985).

Gap junctions are present during early follicular development (Mitchell & Burghardt, 1986) but antrum formation is associated with an increase in the size and number of junctions in response to oestrogen and FSH (Merk et al., 1972; Burghardt & Anderson, 1981). These junctions are most

prominent between granulosa cells resting on the basement membrane separating the theca and granulosa cells. As the antral follicle matures more extensive gap junctional contacts develop between the granulosa cells and between the cumulus cells and the oocyte (Albertini & Anderson, 1974). Metabolic cooperation between cumulus granulosa and the oocyte has been studied by several groups (Heller & Schultz, 1980; Heller, et al., 1981; Moor et al., 1980; Brower & Schultz, 1982). Heller et al. (1981) studied the importance of metabolic coupling between the mouse oocyte and granulosa cells *in vitro*. They reported increased uptake of radiolabelled compounds in granulosa-enclosed oocytes compared with denuded oocytes. Metabolic cooperation was reversibly disrupted by treatments aimed at uncoupling gap junctions. The authors suggest that 85% of metabolites found in the follicle-enclosed oocyte were derived from the granulosa cells. Donahue & Stern (1968) have shown that granulosa cell production of pyruvate supports oocyte maturation *in vitro*. Uncoupling of the oocyte and cumulus cells with loss of junctional contact is a feature of preovulatory maturation (Motlik et al., 1986; Salustri & Siracusa, 1983; Albertini, 1980; Gilula et al., 1978) but the role of this in triggering initiation of meiosis remains unsettled.

The theca of the antral follicle differentiates into two distinct elements - theca interna and externa. The outer layer, the theca externa consists of fibroblast-like cells forming 1-3 layers around the follicle (hamster - Martin & Talbot, 1981). The theca interna, the inner layer, features polyhedral or elongated cells with the morphological features of steroidogenic cells. The theca interna develops a rich network of capillaries and lymphatics. Autoradiographic studies have demonstrated LH receptors in the theca and under LH stimulation the theca develops its full steroidogenic potential producing mainly androgens, some of which are

precursors for granulosa oestrogen metabolism (Magoffin & Erickson, 1981; 1982). Although the stroma/theca cells have received less attention than granulosa cells they may be as important in regulating follicular development and function (Koninckx, 1981). Some *in vitro* models for theca differentiation and function are available (see Erickson, 1983). As will be discussed in the next chapter stromal-epithelial interactions are important in the morphological differentiation of a range of reproductive tissues and a report by Kuroda et al., (1988) indicated that stromal cell defects may be the cause of growth arrest in the follicles of Sl/Sl^t mice.

The Mature Graafian Follicle, Ovulation, and the Corpus Luteum

In this final stage of follicular development meiosis is resumed and the follicle is prepared for ovulation. The granulosa cells have completed their differentiation and the mural and cumulus populations are distinct. The cumulus granulosa cells are coupled to the oocyte, other cumulus cells and surrounding mural granulosa cells by gap junctions. In response to the preovulatory surge of gonadotrophins conformational changes occur in the granulosa compartment. The mural granulosa cells lose their intercellular connections, both gap junctions and desmosomes. The cumulus cells undergo a rapid expansion, progressively losing their communication with the oocyte and with each other, resulting in a loosening of the previously compact cumulus mass. The expansion of the cumulus oophorus is in response to FSH and occurs due to mucification. The synthesis of hyaluronic acid by cumulus cells is essential for expansion (Eppig, 1979a; Salustri et al., 1989). The role of granulosa cells and oocyte in this process has been extensively investigated *in vitro* by Eppig and others (Eppig, 1979a, b; 1980; Salustri et al., 1989; Buccione et al., 1990; Vanderhyden et al., 1990) Of particular interest is importance of the

oocyte in FSH-stimulated expansion as investigated *in vitro* using cumulus-oocyte cultures. Preovulatory hyaluronic acid production by the cumulus cells is inhibited by the removal of the oocyte or ooctectomy, (Buccione et al., 1990).

The mechanism and control of ovulation is beyond the scope of a review of follicular growth and development, Espey (1980) has put forward the hypothesis that it is an inflammatory reaction and recently the role of plasminogen activator has received much attention. Other reviews emphasise a multifactorial hypothesis (see Lipner, 1988) in which prostaglandins may have a significant role. The corpus luteum is formed when the cells of the theca interna and the mural granulosa cells undergo a morphological transformation termed luteinization. Luteinization is the final stage of differentiation of these follicle components. The primary function of the corpus luteum is to produce progesterone, although other hormones including oestrogens, relaxin, oxytocin and prostaglandins are produced by some species, progesterone is a prerequisite of establishing pregnancy.

Expulsion of the oocyte results in the disruption of the follicle and the basement membrane, and blood vessels arising in the theca interna invade the follicle. The main steroidogenic cells of the corpus luteum are the large-luteal or granulosa-lutein cells derived, as the name suggests, from the granulosa layers. These are large, spherical or polyhedral cells containing the cytoplasmic organelles associated with steroidogenesis - numerous mitochondria, an abundant smooth ER, and an extensive Golgi complex. The process of luteinization and the role of the corpus luteum represent yet another example of the functional and morphological differentiation which makes the ovarian follicle such an interesting biological system but lie beyond the remit of the present discussion. Guraya (1985) provides a recent review of the early stages of luteinization

and Niswender & Nett (1988) an overview of the control and function of the corpus luteum.

Ovarian vascular supply and lymphatics

In most species the main arterial supply to the ovary is via the ovarian and uterine arteries (Gillet et al., 1980). The ovarian artery may branch before entering the ovary and in many species, including rabbit and man, the artery and its branches are thrown into spirals or coils (Reynolds, 1950). Within the ovary there is extensive branching and many anastomoses are formed. The ovarian vessels are very tortuous and ovarian veins wind among the arterial coils. The microcirculation of the ovary undergoes marked changes during the fertile period from puberty to the menopause. The development of the ovarian follicle from primordial to Graafian, from ovulation to involution of the corpus luteum causes corresponding development and regression of the architecture of the microcirculation.

Primordial and preantral follicles are avascular insofar as blood vessels do not transverse the follicle basement membrane. The theca interna and externa develop extensive capillary networks which may communicate. The granulosa cells remain avascular until blood vessels invade during ovulation or atresia (Bellman et al., 1953). Kranzfelder & Maurer-Schultze (1989) report the initiation of angiogenesis in the rabbit theca interna only after the membrana granulosa becomes multilaminar. Thereafter the density of capillaries increased linearly with follicle size. These authors, using autoradiography, demonstrated that fluctuations in the granulosa cell labelling index was followed several hours later by a corresponding fluctuation in the labelling index of endothelial cells, suggesting a role for granulosa cells in regulating growth of the thecal capillary network.

The rapid growth of the ovulatory follicle and corpus luteum results in an increased blood flow to the active ovary (Baird & Fraser, 1974) and Niswender et al. (1976) demonstrated, in the sheep, a four-to-six fold increase in blood flow to the ovary containing the corpus luteum compared to the non-corpus bearing ovary. Murakami et al. (1988) studied the vasculature of the rat ovarian follicle using a methylacrylate casting medium. They reported that as the follicle enlarges the network of capillaries grows. The vascular wreath around the Graafian follicle consists of a single layer of sinusoidal capillaries. After ovulation the vascular wreath regresses and the forming corpus luteum develops a new plexus of dilated, sinusoidal capillaries. The mature corpus luteum features smaller calibre, non-sinusoidal capillaries. As the corpus luteum degenerates the vascular supply regresses until only a few small, scattered capillaries remain. The corpus albicans contains few blood vessels.

The lymphatic drainage of the rabbit ovary has been studied by several workers. Taher (1964) reports large lymphatic vessels around multilaminar rabbit follicles and Otsuki et al. (1987a; b) have described the development of the lymphatic system around the rabbit corpus luteum. The lymphatic vasculature changes with reproductive state and aging (Jdanov, 1960; Otsuki et al., 1986) and the ovarian lymph has been shown to contain ovarian steroids - more than twice the level of progesterone than ovarian venous blood (Linder et al., 1964). Linder et al. (1964) demonstrated 100-600ml/hr/100g tissue of lymph was produced by sheep ovaries with a corpus luteum. A role for the ovarian lymphatics in reproductive function seems likely but remains to be fully investigated. Nagle (1987) suggested that the ovarian lymphatics may provide an alternative route for transport of hormones to target tissues in the pelvis.

Innervation

The majority of nerves identified in the ovary are autonomic adrenergic (Rosengren & Sjöberg, 1967; Burden, 1972), although parasympathetic cholinergic nerves have been detected (Burden & Lawrence, 1980) and the density of innervation varies with species: the cat ovary is highly innervated, the human ovary moderately innervated and the monkey sparsely innervated. Nerves usually accompany blood vessels and smooth muscle (Osvaldo-Decima, 1970) and according to Owman & Sjöberg (1966) are prominent near follicles. The ovarian nerves are unlikely to have a major influence on function as shown by the normal cycling in animals with transplanted ovaries and after ovarian nerve ablation but a degree of local modulation or 'fine tuning' is possible. Adrenergic regulation of ovulation and compensatory ovarian hypertrophy are areas of interest (see Burden & Lawrence, 1980 for a fuller discussion).

More recent studies indicate that neurotransmitters, in particular the catecholamines, may have a role in follicle function. β -Adrenergic receptors have been demonstrated in the granulosa cells of immature rats (Aquado, et al., 1982), and catecholamines have been identified in follicular fluid (see above). However follicular cells seem relatively insensitive to catecholamines (rat - Rani et al., 1983; pig - Birnbaumer et al., 1976; rabbit - Hunzicker-Dunn, 1982) but the corpus luteum is more responsive (e.g. Condon & Black 1976). Data on the role of catecholamines in the follicle are at present sparse and conflicting but a stimulatory role in the corpus luteum is possible (see Tsafiriri, 1988 for a review).

Interstitial and other cell types

A number of other cell types and structures are found in the ovary. These include smooth muscle (see DiDio et al., 1980), interstitial

cells, sometimes referred to as the interstitial cell gland (Mossman & Duke, 1973), and embryonic tissue residues (the most clearly recognised of these is the rete ovarii, Harrison & Weir, 1977). The origin of the interstitial cells is uncertain and probably very species variable but two main origins seem likely. They may arise from ovarian stromal cells and from the theca interna of atretic follicles (Guraya, 1977). Interstitial cells are common in some species but difficult to identify in others (Harrison & Weir, 1977). In the rabbit the interstitial cells occupy a major proportion of the ovary (Claesson & Hillarp, 1947). The interstitial cells are steroid producing and their function is influenced by hCG, LH, PMSG and possibly prolactin (Guraya & Motta, 1980). Guraya (1978) presents a comprehensive review of many aspects of the mammalian interstitial cell.

Summary and Aims of this thesis

This introductory chapter has emphasised that folliculogenesis is a complex, progressive process of growth, differentiation and interaction of the three cell types in the follicle: oocyte, granulosa cells and stroma/theca. The process of differentiation depends on the interaction of all three follicular elements modulated by a number of endocrine, paracrine and autocrine mechanisms. Physical as well as chemical interactions between the cell types are probably critical. Vascular and neural influences may also be important modulators of follicle development. The early stages of follicular development and the factors regulating major transitions such as follicle recruitment, induction of the theca and antral formation are difficult to investigate *in vivo* and have been under-investigated compared with the antral stages.

The aim of this thesis was to develop mainly *in vitro* methods for

investigating particular aspects of small follicle development i.e.

- 1) To develop a method for isolating intact preantral small follicles.
- 2) To develop a culture system capable of supporting follicular development *in vitro*.
- 3) To investigate the role of endocrine and paracrine factors in small follicle development *in vitro*.
- 4) To develop a method for studying the role of cell-cell interaction in small follicle development *in vitro*.

In the next chapter the initial attempts to develop suitable methods will be described and aspects of cell biology which may influence *in vitro* differentiation of reproductive tissues will be discussed.

CHAPTER 2

Establishing the culture system.

Introduction

In this chapter some of the preliminary steps in establishing suitable methods for the investigation of small follicle development *in vitro* are described. Approaches to the culturing of follicles and follicular tissues are discussed as are the factors influencing the methods ultimately adopted for this study. The first section will briefly review *in vitro* systems including organ culture, perfusion culture, monolayer cell culture and follicle culture. Then the importance of cell-substrate interactions and the possible influence of epithelial-mesenchymal interactions will be discussed. Finally some preliminary experiments to establish methods of follicle isolation, follicle culture, and the separation of follicle stages are presented.

Approaches to the *in vitro* study of follicle development.

There are four main techniques that can be employed to investigate follicle biology *in vitro*: organ culture of whole ovaries or ovarian fragments, perfusion preparations of whole ovary or ovarian tissue, culture of particular ovarian cell types e.g. granulosa cell cultures, and follicle culture. Follicle culture attempts to culture the complete follicle or a substantial part of the follicle (e.g. granulosa-oocyte complexes) and maintain cellular relationships within the follicle.

Organ Culture.

Organ culture, the maintenance of complete ovaries or ovarian fragments *in vitro*, is one approach which has provided valuable insights into follicular development. Martinovitch (1938) used whole-organ cultures of fetal and post-natal ovaries from rats and mice to study germ cell

development *in vitro*. The ovaries were cultured for up to 4 weeks and the results demonstrated development of the oocytes and refuted neo-formation of germ cells from the germinal epithelium *in vitro*. Ingram (1956) using fetal and adult ovaries from the same species confirmed Martinovitch's findings. Blandau et al. (1965) cultured murine ovarian fragments for more than 50 days but reported frequent oocyte degeneration although some oocytes developed to diameters of 100 μ . Others have used organ culture of varying periods to investigate the effects of hormones on follicle and oocyte development and function *in vitro* (Fainstat, 1968; Neal et al., 1975; Neal & Baker, 1975; Neal et al., 1976; Tyler et al., 1980; Avila et al., 1987).

Ryle (1972) reported antrum formation in organ culture of mouse ovaries when FSH was added to the culture media. Chapekar et al. (1966) cultured murine ovarian fragments on plasma clots and after 15 or 30 days transplanted the fragments to the anterior chamber of the eye of ovariectomized hosts. All host animals receiving fragments cultured for 15 days before transplantation demonstrated oestrogenic effects on vaginal epithelium, uterine weight and mammary duct proliferation. Only 40% of the fragments cultured for 30 days stimulated these tissues in the hosts. Jacobs (1963) cultured mouse ovaries in basal media or media supplemented with HCG and prolactin and then transferred the ovaries to the pectoral muscle of the castrated host. The hormone treated ovaries produced more functional ovarian tissue after transplantation than the control ovaries. Organ cultures have also been used as a model for follicle atresia (Odor & Blandau, 1973).

Perfusion Culture.

The *in vitro* perfused ovary is another preparation which has been used to study the preovulatory follicle. Factors controlling ovulation in the perfused ovary of rat and rabbit ovulation have been investigated by

a number of workers (e.g Lambertsen et al., 1976; Wallach et al., 1978; Kobayashi et al., 1983). Steroidogenesis and the effects of factors such as the gonadotrophins have also been studied using this method (D'Amato et al., 1981; Hedin et al., 1983; Peluso et al., 1984). Other authors have harvested the ova from perfused ovaries to assess the effects of treatments on maturity and fertilizability (Yoshimura et al., 1986; 1987; Brannstrom et al., 1987). The perfusion approach has also been utilised for an investigation of progesterone production by the immature rat ovary (Urbanski & Ojeda, 1985). The perfusion approach is used for short term incubations and shares with organ culture the problem of possible diffusional limitations which would limit its application in studies of sustained growth of small follicles *in vitro*.

Monolayer Cell Culture.

Monolayer culture of isolated cells is one of the classical approaches to cell culture. This approach has produced significant advances in the field of endocrinology particularly the development of the concept of cellular endocrinology (Barnes et al., 1987). Investigations of ovarian physiology have also used the technique extensively. Monolayer cultures have been used to study ovarian surface epithelium (Osterholzer et al., 1985), granulosa cells (Hsueh et al., 1983) and theca cells (Koninckx, 1981). Monolayer co-cultures have also been used to investigate the role of cellular interactions between granulosa and theca cells (Goldring & Orly, 1985). Granulosa cell function and the process of luteinization has been particularly well studied using monolayer cultures of cells from immature hypophysectomized rats (see Hsueh et al., 1983; Erickson, 1983). Granulosa cells are easily aspirated from antral follicles and luteal cells can be obtained by mechanical or enzymatic treatment of dissected corpus lutea.

Cell-substrate interactions can also be explored by coating the culture surface with the appropriate material, eg collagen, laminin or fibronectin. The 'new endocrinology' - that is the recognition that hormones and the more recently recognised growth factors may have important local, paracrine and autocrine, functions - has largely developed due to the cell culture approach.

This approach has severe limitations when applied to the investigation of factors regulating small follicle development. Loss of the structural integrity of the follicle is an obvious problem. The normal structural relationship between oocyte and granulosa cells (and theca in larger preantral follicles) is disrupted as cells spread across the tissue culture surface. Features such as cell shape and intercellular junctions including gap junctions are modified in monolayer culture.

Cell shape, perhaps due to associated changes in the cell cytoskeleton, can alter cell responsiveness to hormones and growth factors and can also reduce differentiated cell functions such as protein secretion. This is particularly true of epithelial cells which are highly differentiated cells both in structure and function. Such cells often have a polarity reflecting their secretory or transport functions e.g kidney tubule cells, thyroid follicle cells and the granulosa cells lining the antral cavity. Many epithelial cells are closely associated with a basal lamina or found in organised layers, for example granulosa cells or the epidermis. They are often responsive to changes in the hormonal environment, eg vaginal or uterine epithelium and may also produce hormones. Maintaining all the differentiated structural, secretory and hormone responsiveness of epithelial cells in monolayer culture has been a major challenge which has been met with varying and partial success. In monolayer culture many epithelial cells undergo some degree of de-differentiation - hormone,

especially steroid hormone responsiveness is altered or lost and production of secretory products such as proteins is reduced. The importance of cell shape and interactions with extracellular matrix is discussed in more detail below.

Follicle Culture.

The final approach considered and adopted for the present study was intact follicle culture. This involves a limited mechanical or enzymatic disaggregation to release the follicle from surrounding tissues with minimal disruption of the follicular unit. The follicles can then be cultured and studied *in vitro*. To develop the method two major problems had to be addressed. Firstly a method was needed to free follicles from the ovarian stroma with minimal disruption of the follicular unit and secondly the culture system had to maintain the normal follicular shape.

A number of workers have developed procedures to isolate ovarian follicles. Grob (1964; 1969; 1971) used pronase to disperse the cellular elements of rat and mice ovaries. The ovaries were incubated in a 0.5% solution of pronase in a calcium and magnesium-free balanced salt solution. As the enzymatic disaggregation proceeded samples were taken and examined microscopically. Pronase treatment of ovaries from immature animals initially yielded single whole follicles. Disaggregation of the follicle unit was reported to occur only after its release from the ovary. Ovaries from older animals yielded follicles in all stages of development. Corpora lutea were released first then large antral follicles. The large antral follicles were more susceptible to dispersal than small antral and preantral follicles. Grob (1964) reported that other enzymes including trypsin, chymotrypsin, pancreatin, collagenase and elastase dispersed the ovaries without the initial release of whole follicles.

Nekola & Nalbandov (1971) also used pronase to disperse rat ovaries. They produced a suspension containing intact follicles, broken follicles, cell clumps and fully dispersed cells. Nicosia et al. (1975) investigated the use of mechanical and enzymatic techniques for isolation of rabbit ovarian follicles. They reported the highest yield and least cellular damage after 20 minutes incubation in 0.5-1 mg/ml collagenase followed by repeated pipetting. Pronase produced a lower yield and caused more disruption of the follicular unit. Eppig (1977) also used collagenase to disaggregate mouse ovaries for culture. Intact larger antral follicles can be obtained by dissection under a dissecting microscope (e.g. Szoltys et al., 1982). This technique is useful in the study of large antral and pre-ovulatory follicles and is also useful as a method of removing larger follicles which would be damaged by mechanical or enzymatic disaggregation. However it is time-consuming and the early preantral follicle is too small for isolation of large numbers by dissection.

Although organ culture was not adopted as the method for this study some pilot experiments were undertaken to assess this approach. These are briefly summarised below.

Another approach to follicle culture, involving the isolation of intact granulosa cell-oocyte complexes or cumulus oocyte complexes, has been extensively used by Eppig and others to investigate later stages of granulosa development, preovulatory cumulus expansion and oocyte-cumulus cell interactions (Eppig 1979a,b; Buccione et al., 1990; Salustri et al., 1989; 1990; Vanderhyden et al., 1990).

The role of substrate

Cell shape and cell-substrate interactions can exert a profound influence on differentiation and function (Folkman & Moscona, 1978; Betz

& Hall, 1987). A number of substrate or phase options are available including glass, plastic, agar, collagen or liquid suspension culture. The glass or plastic substrate can also be modified by treatment with attachment factors such as serum, laminin, fibronectin and collagen. Experience with cells in monolayer culture suggests that two aspects of cell-substrate interaction are probably important in controlling cell behaviour: 1) adhesion to the substrate and 2) specific interactions with elements of the substrate which may alter expression of differentiated function.

A few selected cell types such as haematopoietic cells and tumour cells will proliferate in suspension but adhesion to a substrate seems to be essential for most untransformed cell lines to spread and proliferate in culture (Folkman & Moscona, 1978; Fisher & Solursh, 1979). The second aspect of cell-substrate interaction - specific interactions with elements of an extracellular matrix (ECM) such as basement membrane, or with adjoining cells (Auerbach & Grobstein, 1958) plus the maintenance of cell shape - were judged to be critical for the follicle. As reported below culture on a flat substrate caused disintegration of the follicle unit and the granulosa cells lost their normal morphology, becoming flattened as they spread over the surface of the culture well. This loss of shape could have important implications for differentiated function. Cell-ECM interactions, with resultant mechanochemical effects, may influence morphogenesis and differentiation in a large number of tissues and organs. Embryogenesis, organogenesis, malignant transformation and tissue regeneration are all influenced by mechanical and chemical aspects of interaction with ECM (Kucera & Monnet-Tschudi, 1987; Montesano, 1986). Interactions with the substrate can alter many aspects of cell function including gene expression (Bissel & Barcellos-Hoff, 1987), response to hormones (Tokiwa

et al., 1987), and response to other cells (Ekblom et al., 1980).

Substrate was judged to be of possible importance in follicle culture on three main points:

- 1) Cell shape influences cell function and differentiation.
- 2) The follicle consists of two, and later three, different cells types.

Disruption of the spatial/functional relationships between these cell types can be expected to inhibit normal development.

- 3) ECM, such as the basal lamina, can exert a regulatory influence on cell function and differentiation.

Bissel & Barcellos-Hoff (1987) have investigated the influence of ECM on mammary epithelium and concluded that the functional unit should be considered to be not the cell alone but the cell plus its ECM. Granulosa cells and oocytes might be equally dependent on their ECM. Disruption of the oocyte's specialised ECM, the zona pellucida, has been shown to affect normal folliculogenesis (Skinner et al., 1984). Some aspects of cell-substrate interactions influencing the final choice of substrate are discussed below.

Folkman & Moscona (1978) have shown that cell shape is tightly coupled to DNA synthesis. In three cell types, bovine endothelial cells, WI-38 cells and A-31 cells, incorporation of [³H]thymidine was inversely proportional to the height of the cell (i.e. degree of rounding). Betz & Hall (1987) have demonstrated an effect of cell shape on DNA synthesis and steroid production using Y-1 adrenal tumour cells. Three different substrates were compared - poly(2-hydroxyethyl methacrylate), tissue culture plastic and polylysine. Plastic and polylysine substrates provide a high density of positive charges with which the cells can interact, poly(2-hydroxyethyl methacrylate) is a hydrophilic gel with no net charge. Cells cultured on

poly(2-hydroxyethyl methacrylate) were more rounded in shape and produced higher concentrations of 20 α -hydroprogesterone than the flatter cells grown on plastic or polylysine. The synthesis of the steroid was dose-dependent, higher concentrations of poly(2-hydroxyethyl methacrylate) resulted in greater steroid production which also correlated with a greater degree of rounding, as measured by cell height, of the cells. Poly(2-hydroxyethyl methacrylate) also produced a dose-dependent reduction in [³H]thymidine incorporation into DNA.

Rounding of liver cells in high density cultures produces a similar pattern, with increased metabolic activity but decreased synthesis of DNA (Nakamura et al., 1983). Tokiwa et al. (1987) demonstrated increased responsiveness to hydrocortisone in primary culture of fetal human liver cells on collagen coated substrate. Cells cultured on collagen were more cuboidal in shape and secreted larger amounts of albumin and α -fetoprotein in response to hydrocortisone. Cells on a gelatin substrate produced more protein than on plain plastic but much less than on collagen.

An example of a mechanochemical effect of ECM on maintenance of cell shape and differentiated function is demonstrated by skeletal myotubules in culture. The use of collagen-coated substrate encourages primary cultures of skeletal muscle cells to form myotubules but the myotubule contractility is difficult to maintain as the myotubules tend to become detached from the substrate with a loss of muscle tension and a resultant decline in cellular protein (Vandeburgh et al., 1988). Vandeburgh et al. (1988) reported that embedding the myotubules within a collagen gel matrix maintained the differentiated, morphological characteristics of the cells, increased the total protein in the cell and improved the accumulation of myosin heavy chain. Contractility was also preserved.

The influence of cell shape and interaction with ECM is important in a number of epithelial cells. The importance of a three-dimensional ECM on *in vitro* morphogenesis has been reported for a range of tissues. Disaggregated fetal rat lung cells, a kidney-derived epithelial cell line, rat pancreatic islet cells, and rat intestinal crypts cells all formed tubular or vesicular structures reminiscent of their normal *in vivo* architecture when cultured within a three-dimensional matrix (Douglas et al., 1976; Wohlwend et al., 1985; Amory et al., 1988; Montgomery, 1986). Montesano et al. (1983) demonstrated that endothelial cells cultured on the surface of a collagen gel rapidly organised into a capillary-like network when overlaid with additional collagen gel. Tomooka et al. (1985) reported that trypsin isolated mouse seminal vesicle epithelial cells formed three-dimensional cell colonies with a central lumen when embedded in collagen gel. The decision to use collagen gel was particularly influenced by reports on the effects of a collagen substrate on two cell types, thyroid and mammary epithelial cells. The literature on cell-ECM interactions is extensive. Adamson (1982) presents an introductory review of the effects of collagen on cell division and differentiation, Yang & Nandi (1983) on the use of collagen in cell culture and Reddi (1984) on the role of ECM in development.

A paper by Chambard et al. (1981) first drew my attention to the use of collagen gel. The authors cultured thyroid epithelial cells on, or embedded in collagen gel. When embedded in the gel the thyroid cells formed follicle-like structures with the correct epithelial cell polarity ie with the apical pole of the cells being orientated to the interior of the follicular lumen. Pre-formed monolayers of these cells could be converted to follicles by a collagen gel overlay. This formation of spherical structures with cell polarity preserved was reminiscent of the situation in the membrana granulosa of the antral follicle and suggested further investigation of the

use of collagen gel as a substrate.

The literature on the *in vitro* differentiation of the mammary epithelial cell using collagen gel further emphasised the importance of epithelial cell-ECM interactions. Emerman & Pitelka (1977) showed that dissociated mammary cells formed differentiated, alveolar-like structures when cultured on floating collagen gel membranes. The epithelial cells showed polarization, with tight junctions and microvilli at the luminal surface. A basement membrane separated stroma from epithelial cells. The same tissue grown on plastic failed to form these structures and lost the ability to secrete the characteristic milk proteins (e.g. casein and α -lactalbumin). Prolactin receptors were also lost. Lee et al. (1985) demonstrated a 3 - to 10-fold increase in casein mRNA when mouse mammary epithelial cells were cultured on a floating collagen gel compared with a plastic or fixed collagen substrate. *In vitro* collagen gel culture encouraged formation of a spherical-type structure with epithelial and stromal elements separated by a basement membrane and permitted retention of hormone-induced expression of differentiated function in the epithelial cells. It seemed an appropriate substrate to test for follicle culture.

, Recognition of the importance of factors such as cell shape and ECM on ovarian and in particular granulosa cell function led to the initiation of a secondary project investigating the effects of collagen gel substrate on the *in vitro* morphology of granulosa cells obtained by aspiration of antral follicles of the sheep ovary. This project was not continued after the studies reported below appeared but collagen gel was noted to help retain a more epithelioid shape in cultured granulosa cells. A particularly interesting observation was the presence of occasional Call-Exner-like structures (Figure 2.1) when the sheep granulosa cells were cultured within

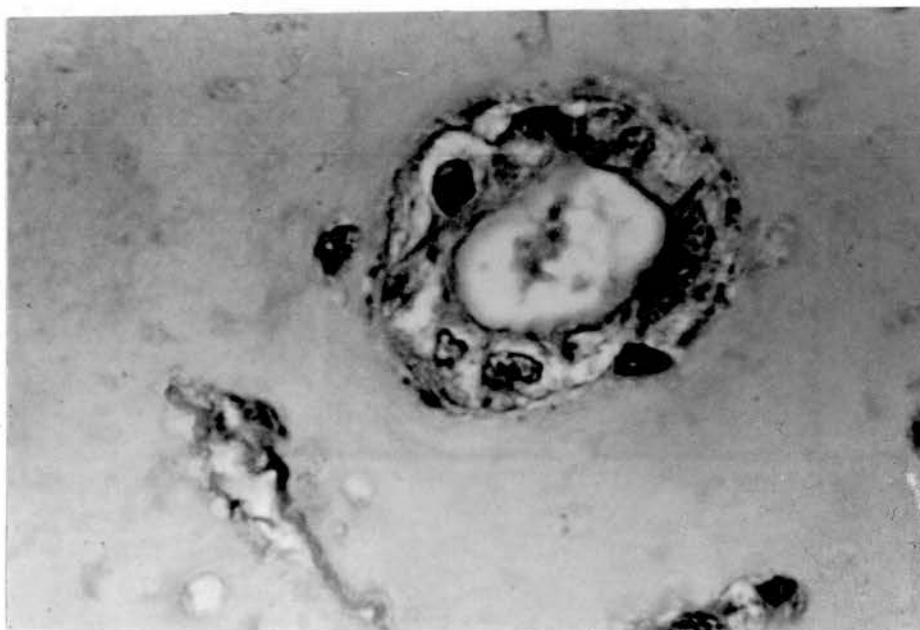


Figure 2.1. Call-Exner-like structure formed by ovine granulosa cells
cultured within a matrix of rat's tail collagen (x160).

a three-dimensional collagen gel matrix (C. Torrance unpublished observations).

The membrana granulosa is delineated by two membranes. The inner membrane, the zona pellucida, separates it from the oocyte while the outer basal lamina separates it from ovarian stroma/theca. As discussed in the previous chapter basal mural granulosa cells may be functionally distinct from antral cells and cumulus cells. Recent studies have examined the hypothesis that ECM may influence granulosa function. Granulosa cells attach more rapidly to culture dishes coated with a bovine corneal epithelium derived ECM and have a more epithelial shape (Furman et al., 1986; Ben-Ze'ev & Amsterdam, 1986). They formed multilayered aggregates of cells. ECM also stimulated a two-fold increase in the number of LH receptors with a parallel increase in cAMP responsiveness to hCG. Progesterone production was elevated but it was negligible in cells grown on untreated dishes (Furman et al., 1986).

Transient rounding up of cultured granulosa cells in response to FSH has been reported (Lawrence et al., 1979; Soto et al 1986). Lawrence et al. (1979) have suggested that alterations in the microfilament component of the cytoskeleton were involved. The involvement of the microfilaments in gonadotrophin regulation of granulosa steroidogenesis is also indicated by Silavin et al. (1984) and Azhar & Menon (1981). More recently Carnegie and colleagues have explored the link between cell shape, cytoskeleton and steroidogenesis in granulosa cells (Carnegie et al., 1987; 1988). Microtubules are one of the cytoskeletal components implicated. Carnegie et al. (1987) reported that agents which disrupt microtubule formation, colchicine and nocodazole, increased FSH-stimulated progesterone production by rat granulosa cells after 24 hours in culture. The cells treated with colchicine and nocodazole were more spherical in shape. A



microtubule stabilising agent, taxol, reduced progesterone secretion. The authors suggest that FSH stimulation results in a controlled depolymerization of microtubules which facilitates transport of cholesterol from lipid droplets into granulosa mitochondria.

Carnegie et al. (1988) furthered their investigation into the role of cell shape by culturing rat granulosa cells in collagen gel. As reported in the preliminary results above using sheep granulosa, the morphology of cells in collagen gel more closely resembled that seen *in vivo*. Carnegie and co-workers reported that the granulosa cells were rounded with diameters only slightly greater than those measured *in vivo*. They secreted nearly three times as much progesterone as granulosa cells in monolayer culture and less of the inactive 20 α -OH-progesterone. An interesting observation from this study was the failure of colchicine to stimulate either progesterone or 20 α -OH-progesterone production by the collagen cultured granulosa cells. It suggests that culturing cells in a three-dimensional matrix of ECM components such as collagen is important not only for retention of normal cell shape but also for maintenance of interactions with the ECM that may provide a more physiological regulation of differentiation.

Epithelial-mesenchymal Interactions.

Hisaw (1947) has suggested that communication might exist between the ovarian stroma and the follicular epithelium and there is some evidence suggesting that stromal defects will inhibit follicle development (Kuroda et al., 1988). Theca-granulosa interactions in steroidogenesis are well established but the possibility of a regulatory role for mesenchymal-epithelial interactions in preantral growth is largely unexplored. Mesenchymal-epithelial interactions are important in morphogenesis and

cytodifferentiation in a number of tissues including lung, skin, thyroid, pancreas, salivary gland, cartilage, bone and teeth (Wessells, 1977; Sanders, 1988). Mesenchymal-epithelial interactions have been extensively studied in reproductive and urogenital epithelial tissues, notably mammary gland, prostatic gland, seminal vesical, Sertoli cells and kidney (Sakakura et al., 1976; Cunha & Lung, 1979; Cunha et al., 1983; Tung & Fritz, 1980; Tung et al., 1984, Sanders, 1988). At present there is no definitive evidence of a regulatory role for stromal-granulosa interactions before thecal formation but this is an area requiring investigation. A number of aspects of stromal-epithelial interactions identified in other reproductive tissues could also be important in regulating follicle development and some examples are presented next.

Regulation of prostatic epithelium is influenced by the amount of stroma present as demonstrated in a study by Chung & Cunha (1982). Epithelium and mesenchyme were obtained from the urogenital sinus of 16-day old rat fetuses. The two tissues were separated and then recombined, varying the amount of either mesenchyme or epithelium. Recombinant tissue was then grafted into host males and the development of prostatic acinar tissue assessed after one month. When the mesenchyme was kept constant and the amount of epithelial tissue varied there was no effect on prostatic tissue development in the graft. However when the amount of epithelium was kept constant the amount of prostatic acinar tissue increased as the mesenchymal mass increased. Daniel & De Ome (1965) report a similar regulation of mammary epithelium by mammary stroma.

Stromal control of epithelial differentiation is demonstrated by vaginal and uterine tissues (Cunha & Fujii, 1981). These authors isolated the stroma and epithelium from three areas, uterus, cervix and vagina, of neonatal mice. Three patterns of recombination were then studied. When

uterine stroma was recombined with vaginal epithelium, the epithelium developed a uterine morphology in the host. Similarly uterine epithelium recombined with vaginal or cervical stroma exhibits a vaginal or cervical epithelial appearance. The stromal-specific induction was functional as well as morphological - uterine epithelium recombined with vaginal stroma exhibited cornification and mucification in response to the host's oestrous cycle. A similar pattern of stromal induction of epithelial development has been demonstrated by the prostate and seminal vesicles (see Cunha et al., 1983). The possibility of induction of granulosa cell differentiation by ovarian stroma is suggested.

Studies of the interactions between the Sertoli cell and seminiferous peritubular cells indicate that mesenchymal-epithelial cell interactions may modulate the functions of both cell types. Tung & Fritz (1980) reported that co-culture of rat Sertoli cells and peritubular myoid cells resulted in the formation of structures with some resemblance to the seminiferous tubules. Such cultures also developed a basement membrane between the cell types and responded to FSH by secreting androgen binding protein (ABP). Sertoli cell only cultures lost the capability to secrete ABP. The morphological response was not specific to peritubular myoid cells, bladder smooth muscle cells had a similar effect. However myoid cells did not interact with kidney epithelial cells. A more detailed investigation of basement membrane formation in myoid-Sertoli cell co-cultures indicated that laminin was produced by Sertoli cells and fibronectin by peritubular cells (Tung et al., 1984). Skinner & Fritz (1988) have identified a non-mitogenic peritubular cell paracrine factor which stimulates protein production (including ABP) by Sertoli cells.

Mesenchymal-epithelial cells may not require direct cell contact: inductive effects may be via components of the basal lamina separating the

cell types. Important ECM constituents include different types of collagen, laminin, fibronectin, various glycosaminoglycans (GAGs) and proteoglycans (see Adamson, 1982). However the evidence for ECM modulation of mesenchymal-epithelial inductive interactions is not conclusive. Branching morphogenesis develops normally in collagen type I deficient embryos (Kratochwil et al., 1986). Sariola et al. (1988) reported mesenchyme induction of branching morphogenesis of embryonic kidney epithelium is blocked by antibodies to a surface glycolipid, disialoganglioside GD₃. This ganglioside is expressed by the mesenchyme.

Thecal/stromal-granulosa interactions may also involve the secretion of paracrine growth factors. McNatty et al. (1984) observed that co-culture of human granulosa cells with theca or stoma resulted in an increase in the number of granulosa cells. Makris et al. (1983) demonstrated that theca-conditioned media stimulated granulosa proliferation. A similar stimulation of granulosa cells was reported when bovine theca and granulosa cells were co-cultured without direct contact (Bendel et al 1988). Secretion of transforming growth factor- β by rat thecal/interstitial cells has been shown by Bendell & Dorrington (1988). Growth factors and follicular function are discussed in chapter 6.

Preliminary experimental approaches to developing a follicle culture system.

The ultimate aim of this project was to develop methods which would make it possible to study normal follicle development *in vitro*. To fulfil this purpose it is necessary to isolate morphologically and functionally intact primordial and preantral follicles and to define culture conditions which would maintain the integrity and developmental potential of the follicles *in vitro*. For folliculogenesis to proceed normally the system

would have to support granulosa cell proliferation and differentiation, thecal differentiation and antrum formation. In this section pilot experiments utilising a number of different approaches are reported, full experimental details for the system of follicle isolation and culture ultimately adopted are given in chapter 3.

Organ culture

The limitations of the organ culture approach have been discussed above and although organ culture was not adopted as the method for this study a pilot experiment was undertaken to assess this approach. This is briefly summarised below.

Materials and Methods

Ovaries were obtained from 6 F₁ mice, aged 8-11 days post partum, and derived from matings between CBA/Ca males and C57Bl/6 females. The ovaries were dissected free of the animal and cleared; then bisected and set in a droplet of collagen gel (details of the collagen gel given below). The bisected ovaries were then transferred to the wells of a Linbro tissue culture plate (Flow Laboratories, Irvine) containing 1 ml of culture medium. All preparatory steps were carried out in HEPES-buffered medium M199 (Gibco, Paisley) and ovarian fragments were cultured in bicarbonate-buffered M199 (Gibco, Paisley). Both media were supplemented with Gentamicin (100 µg/ml, Sigma, London), Amphotericin B (2.5 µg/ml, Sigma), L- glutamine (100 µg/ml, Flow Laboratories, Irvine) and sodium pyruvate (35 µg/ml, BDH, Poole). The culture medium also contained 10% donor calf serum (Flow Laboratories). Sera were heat-inactivated at 55°C for 35 minutes. The bisected ovaries were cultured at 37°C for 4-8 days. pH was maintained in the physiological range by gassing with 5% CO₂. Medium was changed every second day. Six of the wells were supplemented with 20

ng/ml ovine FSH (NIADDK-oFSH-16, NIH, Bethesda, USA). During the culture period the bisected ovaries were assessed by inverted phase microscopy. At the end of the culture period the tissues were fixed overnight in aqueous Bouin's fluid, processed and embedded in paraffin wax. Sections were cut at 7 μ and stained with haematoxylin and eosin. Sections were mounted with DPX.

Results

Follicles, as assessed by inverted phase microscopy and histology, continued to grow during the culture period. No differences were noted between the two groups. Antrum formation did not occur. The organ cultures demonstrated extensive degeneration and necrosis in the centre of the tissue, a common finding in any organ culture system. The embedding of the bisected ovaries in collagen gel worked well insofar as the fragments did not adhere to the bottom of the culture plate and the gel protected the tissue during handling and processing for histology.

Discussion

These results conflict with those of Ryle (1972) who demonstrated antrum formation in response to FSH. However my results were obtained from preliminary, pilot studies using small numbers of animals and organ culture is a very variable technique. The organ culture approach was judged to be of limited use for the present study. Organ culture has the advantage of retaining follicle structure and maintaining cellular interactions but using this approach theca and granulosa cells cannot be separated and their interactions studied by recombination experiments. Additionally due to the size of the ovaries or ovarian fragments there is the problem of diffusional limitations and degeneration or death of tissue in the centre of the organ. Short term organ cultures can be useful but the results of longer organ culture of ovarian tissue are very variable

and this limits the conclusions that can be drawn (Blandau et al., 1965). The use of collagen gel as a vehicle for tissues in organ culture may be more convenient than alternative methods using grids or membranes to support the tissue.

Follicle Isolation

Three approaches to follicle isolation were tested: mechanical isolation, mechanical and chemical isolation, and enzymatic disaggregation. A rapid method which would permit relatively large numbers of isolated follicles was required. Mechanical isolation was tested first because enzymes are known to damage cells particularly cell surface receptors.

Mechanical and chemical isolation of follicles.

Several attempts were made at mechanical isolation using ovaries from immature mice. The ovaries were dissected, cleared and then placed in a watch glass containing Earles Balanced Salt Solution (Flow Laboratories). Under a dissecting microscope the ovaries were teased apart using fine needles. The quantity and quality of the follicles obtained were subjectively assessed using inverted phase microscopy and by viewing the follicles after fixation and staining of the follicle suspension on a polylysine subbed slide.

Mechanical isolation proved to be very time consuming for the number of follicles achieved. The number of follicles produced was low, many of the follicles were only partially isolated, groups of 3-5 small follicles were common. Mechanical trauma, partial stripping of the granulosa layer, was a frequent finding in the larger preantral follicles. With care the clusters of very small preantral follicles could be further reduced but the time taken to produce a few hundred follicles was judged to be too extended for material destined for tissue culture.

Due to the time required to produce sufficient numbers and the degree of mechanical trauma noted mechanical isolation was not pursued. The only other approach to mechanical isolation of intact follicles found in the literature was used by Sotelo & Brauer (1975). They squashed immature mouse ovaries through a 160 μ m brass mesh screen and reported a yield of 900-1000 follicles from 30-40 ovaries. The authors estimated that 20% of the follicle population remained intact after squashing. The mechanical forces involved and low yield reported suggested this would not be an ideal approach.

The next step combined mechanical isolation as described above with chemical disaggregation. The divalent cations, Ca^{2+} and Mg^{2+} are important in maintaining cell-to-cell connections (Borle, 1967). Addition of chelating agents, usually EDTA or EGTA, weakens the intracellular bonds improving cell disaggregation (Anderson, 1953; Melnick, 1955); however continued exposure to chelating agents may disrupt cell metabolism (Waymouth, 1974). Marked morphological injury to the mitochondria was noted in mouse liver cells exposed to 0.1% EDTA (Harris & Leone, 1966). The divalent cations are also essential for the structural integrity of chromosomes. Dispersal of chromosomal material was noted after treatment with low concentrations of EDTA (Mazia, 1954).

Ovaries from immature mice were dissected free, cleared and bisected and placed in a watchglass containing Ca^{2+} and Mg^{2+} -free phosphate-buffered saline with the addition of 0.2% EDTA (Sigma). The ovaries were incubated in this medium for 10 minutes and then in the same medium teased apart using fine needles. The quantity and quality of the follicles was assessed as above and no improvement of yield or apparent quality was noted. The time the follicles were exposed to mechanical and chemical trauma remained the limiting factor. Again the method was very time

consuming for the small numbers produced. An additional problem was that the EDTA appeared to make the follicles more "sticky" they tended to adhere to pipettes and glassware.

Enzymatic isolation of intact follicles

To minimise the period between killing of the animal and placing the follicles into culture and to improve the yield and quality of the follicle enzymatic methods were considered next. The follicles were required for developmental studies and enzymatically induced changes to the cell surface were a concern. However the time taken to obtain relatively low numbers of follicles by the mechanical method and the risk of metabolic or chromosomal injury if EDTA was used suggested enzymatic methods might be a more viable alternative. Since the method was adopted a report comparing follicle respiration between mechanically and enzymatically (collagenase) isolated rat ovarian follicles has confirmed that mechanical isolation is more harmful than enzymatic disaggregation (Carlsson et al., 1986).

A number of enzymes are commonly used to disperse cells for tissue culture which include trypsin, pronase, elastase, pancreatin, chymotrypsin and collagenase (see Waymouth, 1974; Freshney, 1983 for reviews). Crude enzymatic preparations such as pancreatin and crude preparations of trypsin are effective at cell dispersion but may cause intracellular and cell surface alterations. Trypsin and pronase are often used in conjunction with chelating agents or in a calcium and magnesium depleted medium. Trypsin and pronase have been demonstrated to bind to the cell surface with detrimental effects on the glycoprotein coat and cell surface receptors (Poste, 1971; Kono, 1969). Injury to cell surface receptors and the risk posed by the chemical imbalance of the Ca^{2+} and Mg^{2+} -free media

to such highly differentiated structures as the ovarian follicle suggested another enzyme should be sought.

The enzyme adopted was collagenase. Collagenase is a proteolytic enzyme active at neutral pH which is not inactivated by serum factors (Lasfargues & Moore, 1971). Although used in a serum-free medium in this study collagenase can be used in serum-supplemented medium which can be an advantage if deprivation of serum factors may alter the expression of hormone receptors. Collagenase attacks collagen and reticulin but is less damaging to glycoproteins (Mandl, 1961). Collagenase appears to be less injurious to the cell surface receptors (e.g. Waymouth 1974, Kono 1969).

Establishing optimal concentration and incubation time for collagenase.

The concentration of collagenase and incubation time required for disaggregation of the immature mouse ovaries ^{were} ~~was~~ established experimentally. A range of collagenase concentrations and incubation times were tested. However by itself collagenase treatment did not produce many free follicles and mechanical isolation by repeated pipetting was required.

A number of experiments assessing different concentrations and incubation times were carried out. Between 4-10 mice were used for each experiment. The mice were F₁ hybrids, aged 8-11 days post partum, and derived from matings between CBA/Ca males and C57Bl/6 females. After removal from the animal the ovaries were cleared of all adherent tissue under a dissecting microscope and bisected using fine needles. A HEPES-buffered preparation medium (Medium 199 Gibco) was used throughout the procedure. Collagenase (Sigma type 1) concentrations of 1-5 mg/ml in preparation medium were assessed. Incubation times ranged from 10 minutes to one hour. The incubations were carried out at 37°C. As early results indicated that collagenase by itself or with vigorous agitation was unable

to produce a suspension of isolated follicles, mechanical isolation by repeated pipetting was also tested. After each disaggregation the follicle suspension was examined under the dissecting microscope to assess the numbers of free follicles present. Trypan blue exclusion was used on samples drawn from the watchglass as an indicator of cell viability.

An incubation time of 30 minutes at 37°C was adopted as a compromise between sufficient time for penetration and exposure to the enzyme and minimising the time between harvesting of the ovary and placing the tissues into culture. A collagenase concentration of 1.5 mg/ml was the lowest effective concentration for this incubation period when combined with repeated pipetting using gradually smaller diameter pipettes. Pipetting was equally effective when carried out during or after the collagenase incubation, and so to reduce the exposure of isolated follicles to the enzyme it was decided to carry out the pipetting step after the collagenase had been removed. A 30 minute incubation in 1.5 mg/ml collagenase followed by repeated pipetting consistently produced a suspension of isolated, intact follicles although a few clumps and damaged follicles were also present. Follicles and naked oocytes were able to exclude dye although some single cells and clumps of cells took up the dye indicating cell injury. The complete method for isolating mouse ovarian follicles is detailed in the next chapter.

Having achieved an isolated follicle suspension several problems with the handling of the follicles remained. The isolated follicles had a tendency to adhere to the glassware, leading to large losses. Siliconizing the glassware and the addition of deoxyribonuclease (DNase) 1 (Sigma) to the enzyme solution reduced this problem. It also proved difficult to obtain samples containing relatively even numbers of follicles. The follicles are relatively large and quickly settle: simply shaking the vessel is not

sufficient as the largest follicles settle almost immediately. Some form of constant agitation was required. Shaking devices were tested to overcome this problem. These, however, used rotational movements which caused the follicles to concentrate towards the centre of the test-tube. This meant that the first few samples withdrawn contained higher numbers (and larger follicles) than later samples. As all attempts to produce an homogenous suspension of follicles failed it was decided to take advantage of the effects of settling and rotation. The final suspension of follicles was transferred to a watchglass gently rotated to produce a concentrated area of follicles in the centre of the glass. Once settled these follicles can be viewed under the dissecting microscope and approximately even numbers aspirated using a Gilson pipette. Testing the enzymes and identifying and rectifying the apparently minor technical problems in handling the follicle suspension required several months.

In vivo assessment of collagenase isolated follicles.

As the follicles were required for growth studies it was considered important to assess the effect of collagenase and mechanical isolation on the follicle's *in vivo* developmental potential. The method devised involved transplanting the follicles under the kidney capsule of adult hosts. In chapter 4 a detailed assessment of follicle growth after *in vitro* culture and transfer *in vivo* is presented and the transplantation procedure is described in detail.

Materials and methods

Follicles were isolated by incubation in Hepes-buffered medium 199 containing 1.5 mg/ml collagenase for 30 minutes at 37°C. After enzyme incubation the follicles were washed twice with preparation medium to remove the collagenase and freed by repeated pipetting using a Gilson

pipette and increasingly smaller diameter tips. The isolated follicles were suspended in a small volume of medium. For this initial attempt at transplantation ex-breeding CBA females were used as the kidney in these animals is large and more easily manipulated. The mice were ovariectomized (to minimise endogenous oestrogen levels) at least 2 weeks before being used as hosts. All operations were carried out under intraperitoneal tribromoethanol anaesthesia (6.03 g/kg body weight). The left kidney was exposed and a small nick made in the kidney capsule. The capsule was gently raised to allow insertion of a fine Pasteur pipette, the pipette was used to free the capsule from the kidney tissue and approximately 20 follicles, in 50-100 μ l of medium, were placed in the space created. Daily vaginal smears were obtained by lavage to monitor oestrogenisation of the vaginal epithelium. A single mouse was sacrificed at 90 minutes, 24 hours, 5 and 12 days and checked for the presence of follicles. Kidneys with obvious follicles were fixed and processed for histology. At autopsy a check was made to ensure that no ovarian fragments which might account for oestrogenization remained.

The method of follicle culture eventually adopted involved setting and culturing the follicles in a matrix of collagen gel (see below). The collagen gel vehicle was used to repeat the *in vivo* assessment of freshly isolated follicles. Follicles were isolated as before and then suspended in 20 μ l of gel solution. After the gel had set it was transferred to the ovariectomized hosts. Mice were sacrificed between 5 to 14 days. Assessment of follicle grafts by vaginal smears and histological sections was carried out as before.

Results

Pipetting of follicles under the capsule proved difficult, particularly

with larger follicles. A loss of material was evident during the insertion procedure. The mice sacrificed at 90 minutes and 24 hours showed no sign of follicles. Mice at 5 and 12 days had some evidence of grafts and histology demonstrated that some follicles were present. Multilaminar follicles with a thecal layer were seen but no antral follicles were observed. Vaginal smears showed occasional cornified cells from about day 7 but no persistent pattern emerged. The use of collagen gel as a vehicle made it much easier to transfer the follicles. Vaginal smears showed cornification and from day five, grafts contained antral follicles (Figure 2.2).

Discussion

The development of antral follicles and production of oestrogen, as indicated by cornification of the vaginal epithelium, suggested that no irreversible damage to the small follicles had been caused by the disaggregation procedure and that follicle culture could proceed with some confidence.

Establishing the culture conditions

A culture system which would allow the follicular elements to retain their normal 3-dimensional shape and attachments and permit the follicles to grow *in vitro* was required. A number of factors such as the culture medium, choice of supplements and the types of culture vessel and culture surface had to be considered.

A large range of medium are available for tissue culture. The medium adopted was commercially prepared Medium 199 with Earles Balanced Salt Solution. This is a general purpose medium formulated by Morgan et al. (1950). It contains over 60 ingredients covering the full range of

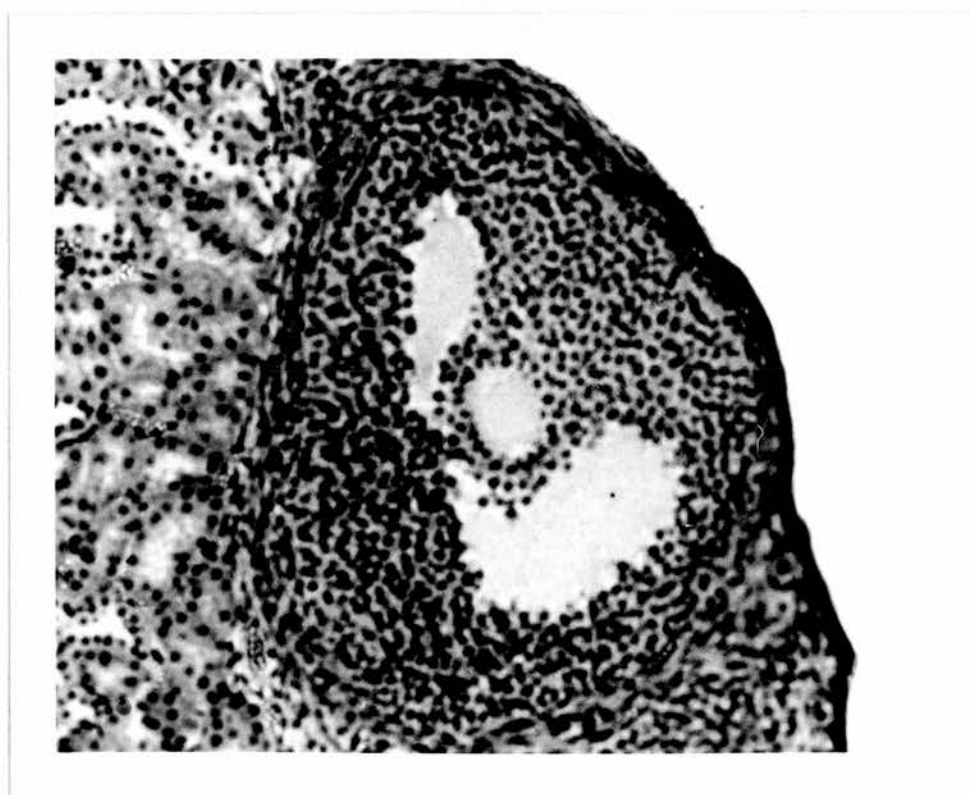


Figure 2.2 Section of a collagen gel containing freshly isolated follicles grafted under the kidney capsule. Section shows an antral follicle with theca. Animal killed 10 days after transplantation (x 160).

inorganic salts, carbohydrate, amino acids and vitamins. It can be used after the addition of methyl cellulose as a suspension medium. Morgan et al. used the medium for extended culture of chick embryos. It has been successfully used for culture of isolated oocytes in this laboratory (R.G. Gosden, personal communication). The medium was supplemented with additional glutamine and pyruvate, an essential substrate for oocyte metabolism (Donahue & Stern, 1968).

As the culture was anticipated to be relatively slow growing and the culture period might extend to 2 or 3 weeks, antibiotic and antifungal agents were used although there are some disadvantages in the use of antimicrobials in cell culture. Gentamicin and fungizone were chosen. Gentamicin is a broad spectrum antibiotic, also active against mycoplasma: and fungizone, the sodium deoxycholate complex of amphotericin B, is active against a wide range of yeasts and fungi. Tissue culture grades of both antimicrobials were used at the manufacturers recommended concentrations. Neither antimicrobials are thought to inhibit cell growth at these concentrations. However some adverse effects of antimicrobials have been reported and the use of fungizone in particular has been reported to alter cultured cell function. Growth hormone and prolactin secretion by a rat pituitary tumour cell line (GH₃) was reduced in the presence of 2.5 µg/ml fungizone (Lapp et al., 1987). Fungizone but not gentamicin reduced progesterone output by chicken granulosa cells in culture (Robinson et al., 1988).

The M199 was used with HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer as a preparation medium and with bicarbonate buffer as a culture medium. HEPES buffer is more robust than bicarbonate but there have been some reports of possible toxic effects of HEPES. Low concentrations of HEPES, between 10 and 15 mM, caused vacuolation in

chick embryo chondrocytes, decreased smooth muscle cell contraction and reduced growth in endothelial cell cultures, possibly by stimulating the production of oxygen radicals (Poole et al., 1982; Altura et al., 1980; Bowman et al., 1985). Higher concentrations (>25 mM) can alter cultured cell structure and function (Brune, 1981; Daniel & Wolf, 1975; Verdery et al., 1981). Due to possible toxic effects and the evidence suggesting that cells in culture require CO_2 as an essential nutrient (Itagaki & Kimura, 1974) a bicarbonate buffered M199 was adopted as the culture medium.

The final consideration with regard to the composition of the culture medium was which serum to choose if serum supplementation was required. As discussed in the review a variety of factors can potentially influence small follicle development and these include hormones and growth factors. Serum is routinely used in cell culture as it provides a rich but undefined source of nutrients, proteins, minerals, hormones and polypeptides which may include growth and attachment factors (Freshney, 1983). Heat inactivation will remove complement from the serum without damaging polypeptide growth factors but other more labile constituents may also be inactivated with adverse effects on cell attachment (Giard, 1987). As the growth requirements of small follicles were unknown it was decided to culture initially in the presence of 10% donor calf serum. This serum was chosen as it would contain less gonadotrophin than fetal calf serum. Serum is likely to be the most variable element in the culture medium and care was taken to ensure that all experiments in a series were carried out using serum from the same batch. The serum was heat inactivated and then dispensed and stored in individual aliquots at -20°C to avoid frequent defrosting. A partially-defined medium for follicle growth is discussed in chapter 6.

Having obtained an isolated follicle suspension and decided on a

suitable culture medium the next element of the culture system to be explored was the culture substrate. The first substrate tested was conventional tissue culture plastic, suspension culture and roller culture were briefly tested but the approach ultimately adopted used a three-dimensional collagen gel matrix.

Follicle culture using a plastic substrate

Materials and methods

An isolated follicle suspension was produced by collagenase disaggregation as described above using the ovaries from 10 immature mice. The final follicle pellet was resuspended in 2 ml of culture medium containing 10% donor calf serum and 0.5 ml of the suspension added to the wells of a Linbro tissue culture plate (Flow Laboratories). The culture medium was changed at 24 hours and then every third day. The progress of the cultures was monitored using phase contrast microscopy.

Results

The follicle suspension contained a mixture of cell types including follicles, denuded oocytes, individual cells and clumps of cells which could be seen floating in the medium. The follicles soon settled onto the plastic substrate and by 24 hours most follicles and other cells in the suspension had attached. The first change of culture medium removed most of the unattached cells. The granulosa cells began to spread disrupting the follicular unit and releasing the oocyte into the culture medium (Figure 2.3). Within 2-3 days all the follicles had been disrupted. The process of follicle disruption was retarded when care was taken to seed all the follicles closely together in the centre of the culture well. However even with closely packed follicles most oocytes had been liberated by 5 days in culture.

Discussion

The disruption of the follicles reported here corresponded well with the observations reported by Eppig (1977) except that he found 65-70% of the oocytes remained attached to the granulosa cells (after 7-12 days) when the follicles were seeded very close together. Eppig also reported some increase in oocyte size over the culture period. An interesting observation was a reduction in the number of fully grown oocytes recovered when the medium was supplemented with FSH or E₂. Fully grown oocytes were able to initiate meiotic maturation when released from the granulosa cells. The loss of three-dimensional structure, and in particular the problems this would impose in studying theca and antral induction was judged to limit the usefulness of this approach and it was not pursued any further. Addition of attachment factors such as fibronectin or laminin might have altered the behaviour of the follicles but it was decided that a three-dimensional matrix rather than two dimensional approach was needed.

Collagen gel culture

Follicle culture represents a stage between conventional cell culture and organ culture and one of the main cell types, the granulosa, are epithelial cells. Tissue culture has long recognised that epithelial cells in particular may require ECM elements to maintain differentiation *in vitro* and a more physiological substrate was sought. The literature emphasised that ECM was important not only as a three-dimensional scaffold maintaining normal cell shape and normal tissue structure but also for its chemical properties which may be equally important in regulating cell function. The paper by Chambard et al. 1981 on thyroid follicles in collagen gel suggested this would be an appropriate approach to test.

Gel solution was produced from rat tail tendons as described in detail in the next chapter. The gel solution was easily prepared and when tested



Figure 2.3 Phase contrast photomicrograph showing follicle cells adhering and spreading over the plastic substrate. Central oocyte is in the process of being shed and part of a free oocyte can be seen in the top right of the picture (x 160).

with dyes appeared to present no barrier to diffusion of small molecules. Electron microscopy of such collagen gels has shown a mesh of straight and branching collagen bundles dispersed within a watery milieu with a slightly greater congregation of bundles in the peripheral 2 μ m of the gel (Elsdale & Bard, 1972).

It initially proved difficult to suspend the isolated follicles within the gel. The gel solution required careful and rapid handling. The collagen was extracted by acetic acid and clarified by centrifugation and the resulting solution was polymerised by adjusting temperature, pH and ionic composition towards the physiological range by the addition of concentrated culture medium, serum and sodium hydroxide. If the solution was not kept ice-cold after the addition of the hydroxide it set within minutes. Simply mixing collagen solution with the follicle suspension proved unsatisfactory.

Firstly, as described above, it was difficult to obtain an even suspension of follicles and agitation of the collagen solution resulted in air bubbles which were difficult to remove. Secondly the follicles had to be concentrated by gentle centrifugation and most of the supernatant drawn off to prevent over dilution of the collagen gel. When the follicles were added to the gel additional pipetting was required to break up the follicle pellet and provide an even distribution within the gel solution. There appeared to be a loss of follicles at this stage. Examination of the pipettes used under a dissecting microscope revealed a thin layer of follicles and set collagen gel coating the inside of the pipette. Additionally the test-tube used to hold the follicle-gel suspension was found to have a thin film of set collagen containing follicles on its inner surfaces. Another problem was that when the collagen-follicle suspension was pipetted out the newly formed gels tended to collapse under their own weight. The wells of

a Terasaki plate were used as a mould to support the gel until polymerisation was complete.

The final solution adopted avoided the problems of obtaining an even number of follicles per gel, dilution of the gel by the follicle suspension and the loss of follicles due to premature setting of the gel. Follicles were concentrated in the bottom of a watchglass as described above and 10 μ l samples transferred to the wells of a Terasaki plate. A small volume of collagen solution was prepared and pH and ionic balance adjusted. The solution was kept ice-cold. A tissue was used to draw off excess fluid from the follicle suspension in the wells and then 10 μ l of the collagen gel mixed with the follicles. Once polymerised this formed a small cone-shaped gel containing embedded follicles.

Initial cultures using these gels revealed two additional problems. During the culture period the gels contracted and follicles were lost into the medium. These follicles soon attached to the bottom of the dish and grew as monolayer cultures. Gels were harvested and fixed in Bouin's fluid for histology. When sections were stained there was less than the expected number of follicles. Again contraction of the collagen matrix was the problem, follicles being lost during processing for histology. Both problems were simply overcome: after the gel-follicle suspension had set another 20 μ l of gel solution was pipetted into a Terasaki well and the set gel transferred into it. This formed a second coating of gel which prevented follicle loss during culture and processing. The methods developed provided a novel approach to the isolation and culturing of mouse ovarian follicles.

Separation of follicles by size

A secondary aim of the project was to develop a method of separating follicles by developmental stage and size. This would enable experiments

looking at homogeneous follicle populations or specific stage-related interactions to be carried out. The suspension produced by collagenase disaggregation was a mixture of isolated follicles of different sizes, some clumps of follicles, damaged follicles, isolated oocytes, clumps of granulosa, clumps of interstitial and stromal tissues, individual cells and remnants of other ovarian components such as blood vessels. The use of graduated nylon meshes was explored as a means of separating follicles. Wigle et al., (1983) used a 1g sedimentation column featuring precisely woven nylon meshes for bulk separation of multicellular spheroids of tumour cells. A similar column was produced by the departmental workshop and used with precision woven nylon meshes (Nybolt ASTM, Simon Ltd, Stockport, Cheshire, UK).

Follicle suspensions were produced from the ovaries of 10-day old mice by collagenase disaggregation. The sedimentation column (Figure 2.4) was primed with ice-cold preparation medium (details later) and the follicle suspension added to the top. The column was emptied from the base after about five minutes and fresh medium added. Sedimentation times ranging from 10 to 60 minutes were tried. The range of meshes tested, size of mesh opening and the percentage of open surface area are given in Table 2.1. To improve movement through the smaller meshes with a low open surface the column was periodically agitated.

The mesh trapped follicles but the size range was wide and partial follicles, clumps of cells, larger free oocytes and other debris contaminated the samples. The fraction produced by the smallest mesh used, 44µm, contained the smallest follicles but also the usual debris and was rich in single cells. Problems were also encountered in releasing material from the mesh screens. The sedimentation tower did permit a degree of follicle separation but contamination by fragments from larger follicles, naked

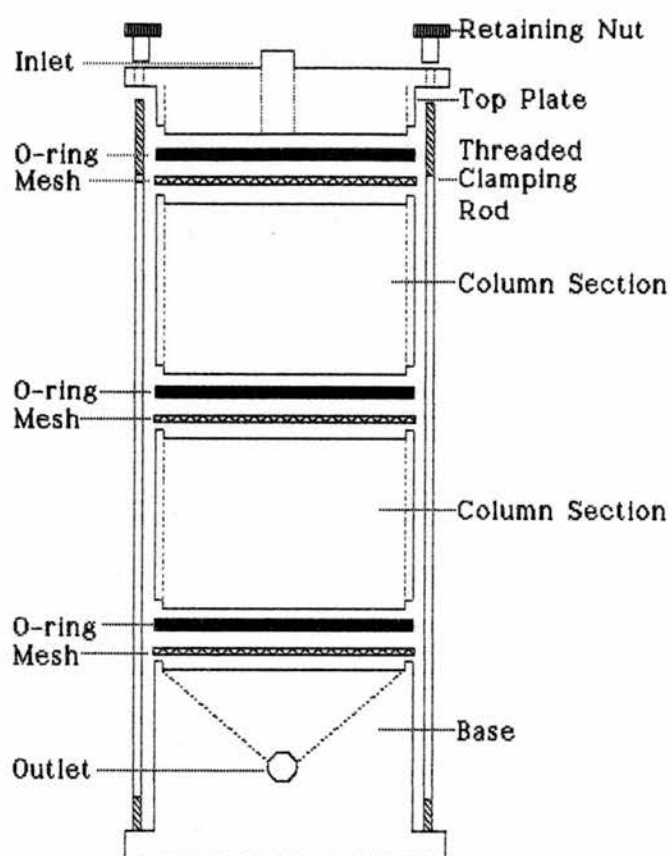


Figure 2.4. Follicle separation column (adapted from Wigle et al. 1983).

Table 2.1 Mesh Screens Tested using Sedimentation Column.

Mesh Number	Mesh Opening μm	Threads per cm	Open Area %
70-210 ASTM	210	28.6	36
100-149 ASTM	149	41.8	39
120-125 ASTM	125	48.8	37
170-88 ASTM	88	67.6	35
325-44 ASTM	44	126.6	31

oocytes and clumps of other cells prevented a pure follicle suspension from being obtained.

Another approach attempted was the use of Percoll, a non-toxic density medium with optimal osmotic properties (Pertoff et al., 1977). Percoll can be used in discontinuous or continuous gradients and has been used to isolate structures such as pancreatic islets as well as free cells (Buitrago et al., 1977). Percoll (density = 1.130 g/ml), manufactured by Pharmacia, was obtained through Sigma (Poole, Dorset, UK). Discontinuous gradients ranging from 40 to 100% Percoll in preparation medium were tested under unit gravity and after centrifugation at between 100 and 500g using a MSE benchtop centrifuge. Cells were carefully layered on top of the Percoll bands in a 15 ml centrifuge tube and left or spun for 5-10 minutes. As with the sedimentation column some separation of follicles was achieved but the mix of follicle size within the bands was still wide and contamination by other tissue fragments occurred.

The main problem encountered with both approaches was the heterogeneity of the follicle population. Follicle diameter did not necessarily correspond with follicle stage, some one layered follicles were as large as some two layered follicles. It had been hoped to obtain discrete bands of follicles of approximately the same size and stage of development for studies looking at the effects of isolating each stage and then recombining them with follicles (or medium conditioned by follicles) of more advanced stages. The variability of follicles in the bands and in particular the presence of oocytes and portions of larger disrupted follicles limited the usefulness of the preparations obtained. Possibly a combination of crude separation using the column and then more precise, perhaps continuous Percoll gradients might have yielded better results.

Conclusion

In this chapter some of the problems and successes of the early stages in the development of suitable methods for isolating and culturing small follicles have been described and some of the factors influencing the methods chosen discussed. A satisfactory procedure combining enzymatic and mechanical isolation was achieved and transplantation of the isolated follicles under the kidney capsule of ovariectomized adult hosts indicated that at least some of the follicles retained their developmental potential. The three-dimensional structure of the follicle was maintained by culturing them in a matrix of collagen gel. The collagen gel also proved to be a convenient vehicle for culturing and handling the follicles after isolation. Attempts to separate the follicles by size and density were not successful. In the next chapter the method of follicle isolation and collagen gel culture is described and the *in vitro* development of preantral follicles investigated.

CHAPTER 3

A quantitative study of the development of isolated preantral
follicles in collagen gel.

Introduction

The ovary of the adult mouse contains thousands of follicles including non-growing primordial follicles, slowly growing preantral follicles and the more rapidly maturing antral follicles. Primordial follicles are continuously being recruited from the non-growing pool into the growth phase. The progression of follicles from primordial towards the antral stage occurs without any further resting phases. The factors initiating follicle growth and regulating progress through the preantral phase are largely unknown. Follicle recruitment and the preantral phase of growth continue during periods of endocrine disruption such as pregnancy and after experimental manipulations such as hypophysectomy. This indicates that the gonadotrophins and ovarian steroids which play a major role in regulating progress through the antral and preovulatory phases of folliculogenesis are not as critical in preantral growth. They may well have a role in the small follicle but other factors are likely to be equally important. Recently, attention has focused on the importance of paracrine and autocrine mechanisms in controlling ovarian function (reviewed by Hsueh, 1986). A number of studies, using mainly *in vitro* approaches, have reported effects on granulosa cell proliferation and differentiation of ovarian steroids (oestrogens, androgens and progesterone), growth factors (EGF, IGF-I, TGF- β , FGF), other hormones and peptides (insulin, VIP, GnRH-like peptides), and possibly neurotransmitters. Other studies have identified the importance of factors such as cell shape and cytoskeleton, cell interactions and ECM-cell interactions.

Knowledge gained from studies of the antral follicle is a useful starting point and may provide some indications of factors which may be important in earlier stages of growth. However such knowledge may not be

reliable when extrapolated to the very different situation of the primordial and preantral follicle. To study these structures and their development it is useful to be able to isolate the follicles from systemic and intraovarian influences, permitting experimental modulation of the physiochemical environment. However to reproduce physiological interactions *in vitro* it is preferable to maintain normal three-dimensional relationships within the follicle unit.

One of the major aims of this research was to develop a method enabling normal follicle structure, function and growth to be maintained *in vitro*. Maintenance of cell shape and structural relationships within the follicle were important considerations and the collagen gel system appeared to provide a suitable 3-dimensional medium for supporting follicle growth. Ideally the system would also provide standardisation of the culture conditions with an equal distribution of follicles, both numbers and stages, within each gel and each culture well. This chapter presents details of the method adopted and reports on the development of isolated follicles over a two week culture period. The effect of follicle numbers on growth over a 6 day period is also investigated. The usefulness and limitations of this method are discussed.

MATERIALS AND METHODS

Animals

The mice used in this study were F₁ females aged 8-11 days post partum and derived from matings between CBA/Ca males and C57Bl/6 females. Breeding pairs were obtained from Bantam & King. Breeding pairs were provided with a pelleted diet and water *ad libitum* and housed under a light/dark cycle of 14 hours light starting at 0700 GMT. Room temperature was maintained at 21°C. The immature mice were used

immediately after their removal from the mother. F₁ hybrids were chosen for hybrid vigour and to maximise genetic uniformity.

Culture Media

All preparatory steps were carried out in N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES) buffered medium M199 (Gibco, Paisley, UK) and follicles were cultured in bicarbonate-buffered M199 (Gibco). Both media were supplemented with gentamicin 100 µg/ml (Sigma, Poole, Dorset, UK), fungizone (amphotericin B) 2.5 µg/ml (Sigma) L- glutamine 100 µg/ml (Flow Laboratories, Irvine, UK) and sodium pyruvate 35 µg/ml (BDH, Poole). The culture medium also contained 10% donor calf serum (Flow Laboratories). Sera were heat-inactivated at 55°C for 35 minutes. Preparation medium had an osmolality of 290-310 mosmol Kg⁻¹ as measured by a Wescor vapour pressure osmometer and a pH of approximately 7.4 in room air and at room temperature. The culture medium had an osmolality within 280-300mosmol Kg⁻¹, pH was maintained in the physiological range (7.2-7.4) by gassing with 5% CO₂ in a humidified incubator. Medium pH was monitored by observation of the colour: the medium contained phenol red which is red at pH 7.4 bluish red at 7.6 and orange-yellow at 6.8. However since colour assessment is highly subjective a range of pH standards (7.0 - 7.6) for colour comparison were made up using sterile balanced salts solution containing phenol red as described by Freshney (1983).

Collagen gel solution

Collagen gel was extracted from rats tail tendons after the methods of Ehrmann and Gey (1956) & Chambard et al. (1981) as follows. The tails were sterilised overnight in 70% alcohol then, beginning at the tip, the tail was gripped between two pairs of artery forceps and fractured into small

sections. Each section with its attached strands of tendon was pulled free and the tendon harvested before making the next fracture. The tendons were transferred to 70% alcohol, and then rinsed in sterile distilled water. 1g of tendon was added to 100ml of 1:1000 acetic acid and stirred at 4°C for 48 hours. The solution was centrifuged at about 1500 G on a MSE centrifuge for 1 hour. The solution could be stored at 4°C for up to eight weeks. Gels were prepared immediately before use by mixing 1.6 ml of collagen solution with 200µl of the required serum and 200µl x10 Medium 199 at 4°C. pH was adjusted by adding 500mM NaOH (100-120 µl) until the indicator (phenol red) turned the appropriate colour for pH 7.2 - 7.4.

Follicle Isolation

All steps were carried out using ice-cold preparation medium and in each experiment 24 mice were used. The animals were killed by decapitation and the ovaries were removed and placed in a watchglass containing medium. All subsequent steps were carried out using sterile techniques in a laminar flow hood. The ovaries were cleared of adherent tissue, transferred to a fresh watchglass and bisected with fine needles. The bisected ovaries were incubated with gentle rotation at 37°C in a 10ml tube containing Hepes-buffered medium with 1.5 mg/ml of collagenase (Sigma type 1) and 40 units/ml of deoxyribonuclease I (DNase I, Sigma). After 30 minutes the ovaries were centrifuged (100-200 G) to pellet the tissue and the supernatant was removed. The pellet was resuspended in 10ml of medium and centrifugation repeated to ensure that all the enzyme solution had been removed. The follicles were then resuspended in 3ml of medium and transferred to a watchglass.

Under a dissecting microscope follicles were isolated by repeated pipetting of the bisected ovaries using Gilson pipettes. As the fragments

decreased in size pipette tips of progressively smaller diameter were used. To minimise mechanical trauma from repeated pipetting freed follicles were harvested frequently. The follicles were collected using a 200 μ l pipette tip and filtered through a 125 μ m nylon mesh (Nybolt ASTM 120-125 Simon Ltd, Stockport, Cheshire, UK) to remove any clumps. The filter was flushed to remove any trapped follicles. When pipetting no longer freed follicles the remaining fragments were discarded. The follicles were pelleted by gentle centrifugation, the supernatant removed and the follicle pellet resuspended in 3 ml of medium.

The follicles were transferred to a watchglass and gently pipetted to ensure an even suspension of isolated follicles. Gentle rotation concentrated the follicles in the centre of the watchglass. 20 μ l samples were pipetted from the central concentration into the wells of a Terasaki plate (Flow Laboratories). The rotation and pipetting was repeated until 16 samples had been obtained. A sterile, paper tissue was used to remove excess fluid from the wells and 10 μ l of the collagen gel solution was added and pipetted once to mix with follicles. The follicles were incubated for 2-3 minutes at 37°C until the gel had set. 20 μ l of gel solution was pipetted into another set of wells and the set 10 μ l gels transferred into these wells. The double gels were allowed to set. This double gelling is necessary to avoid loss of follicles due to contraction of the gels during culture and processing for histology. The sixteen gels were transferred to the wells of a Linbro tissue culture plate (Flow Laboratories).

Four wells containing 2 ml of culture medium were pre-incubated to warm and gas the medium and the gels randomly assigned, 4 to a well. The gel cultures were incubated at 37°C with 5% CO₂ in a humidified incubator. The medium was changed at 24 hours and every third day.

The VHS videotape programme presented with this thesis provides a short, visual guide through each step of the technique.

Microscopy and Histology

Follicles could be monitored during culture using inverted phase contrast microscopy because collagen gel is transparent. At the termination of culture the gels were fixed overnight in aqueous Bouin's fluid, embedded in paraffin wax and sectioned at 5 μ m. Sections were stained with haematoxylin and eosin and mounted with DPX. The protocol for fixing and staining is given in Appendix 1.

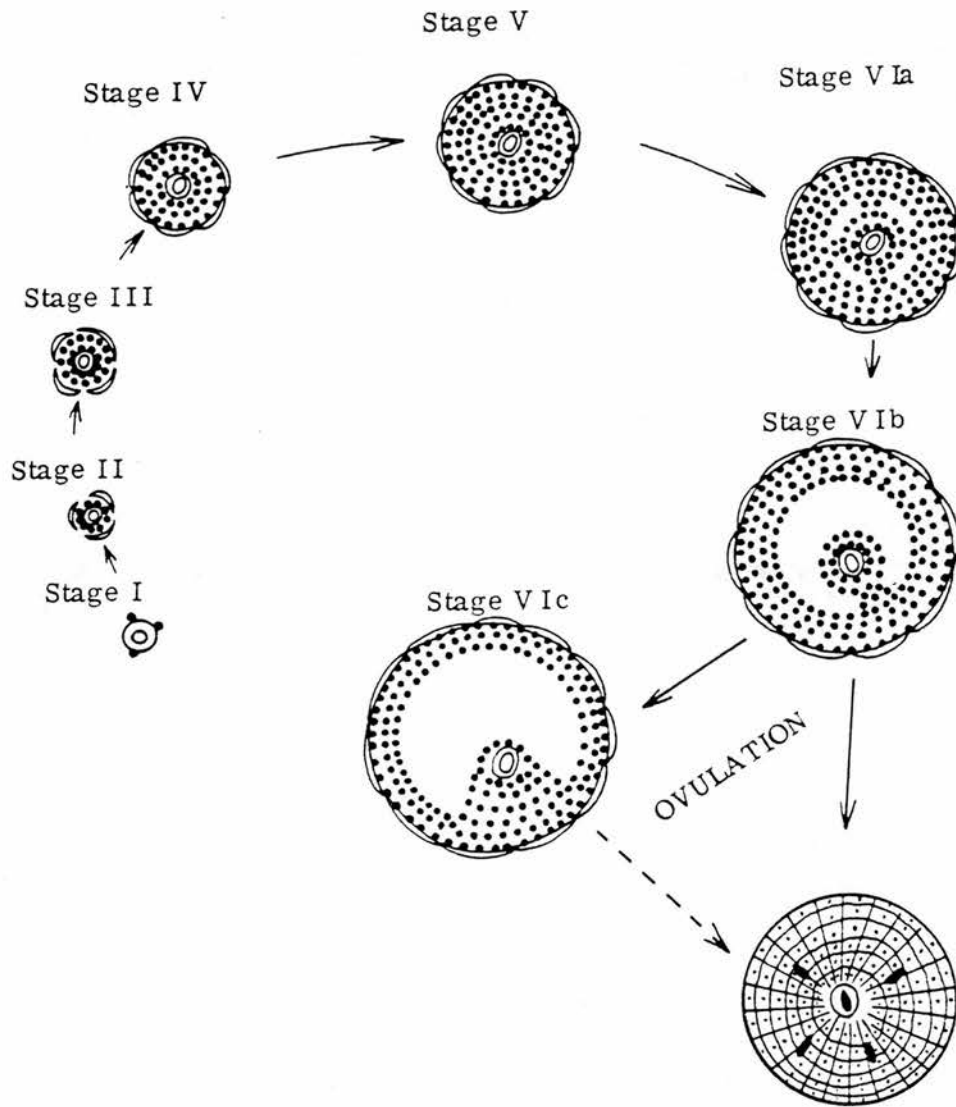
Classification of follicles

Follicles were observed under x400 magnification and classified according to the number of cell layers surrounding the oocyte, based on the established system of Mandl & Zuckerman (1951) - Figure 3.1. Follicles were also classed as either disrupted (including free oocytes and any follicles with a disrupted granulosa layer) or intact (granulosa layer(s) intact). Figure 3.2 shows an example of a disrupted follicle. The quality of the intact follicles was also assessed. Follicles were classed as intact but damaged if they had more than slight contraction of the oocyte from the granulosa layers (Figure 3.3), with nuclear contraction, with obvious separation of the granulosa layers, or with more pycnotic cells than cell layers.

Longitudinal study of follicle growth

Experiments were designed to establish the pattern of follicle growth in collagen gel culture. For each experiment 4 gels from one well were allowed to equilibrate in the incubator for one hour and then harvested for

Figure 3.1. Follicle classification (Mandl & Zuckerman, 1951).



- | | |
|-----------|---|
| Stage I | Oocyte surrounded by a single layer of squamous granulosa cells. |
| Stage II | Growing oocyte surrounded by a single layer of cuboidal granulosa cells. |
| Stage III | Growing oocyte surrounded by two layers of cuboidal granulosa cells. |
| Stage IV | Growing oocyte surrounded by three layers of cuboidal granulosa cells. |
| Stage V | Oocyte surrounded by four or more layers of cuboidal granulosa cells but no antrum. |
| Stage VIa | Antrum forming. |
| Stage VIb | Oocyte surrounded by cumulus mass and a fully formed antrum. |

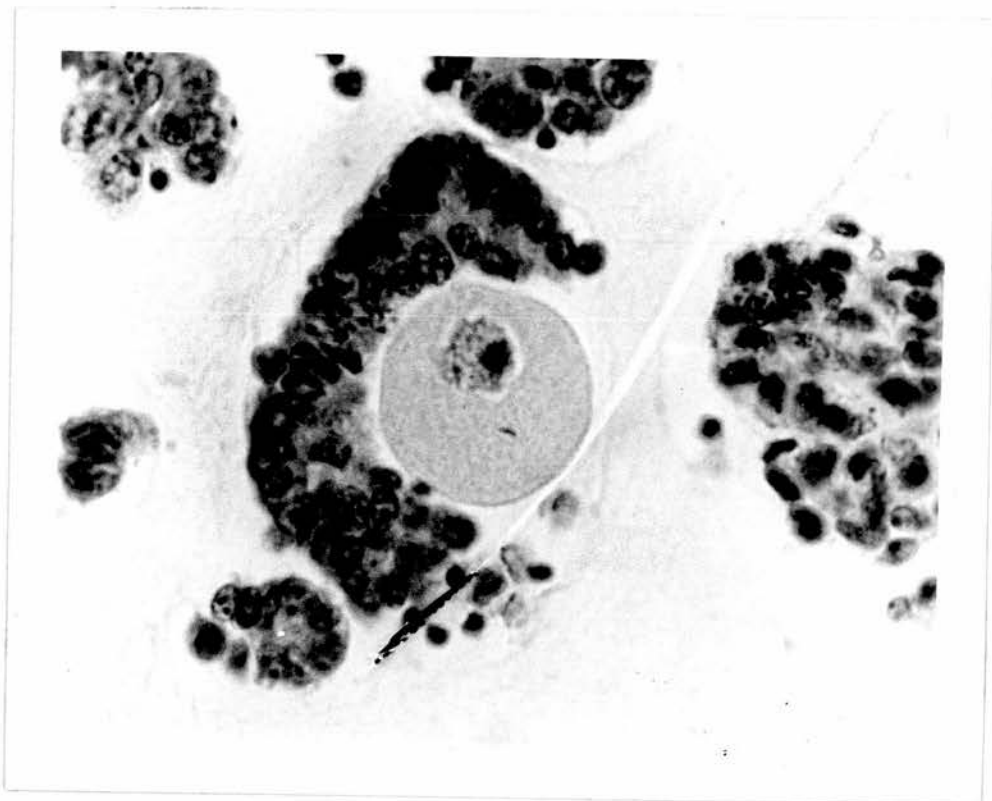


Figure 3.2. Section of 0 hour collagen gel showing a disrupted follicle
(x 480).

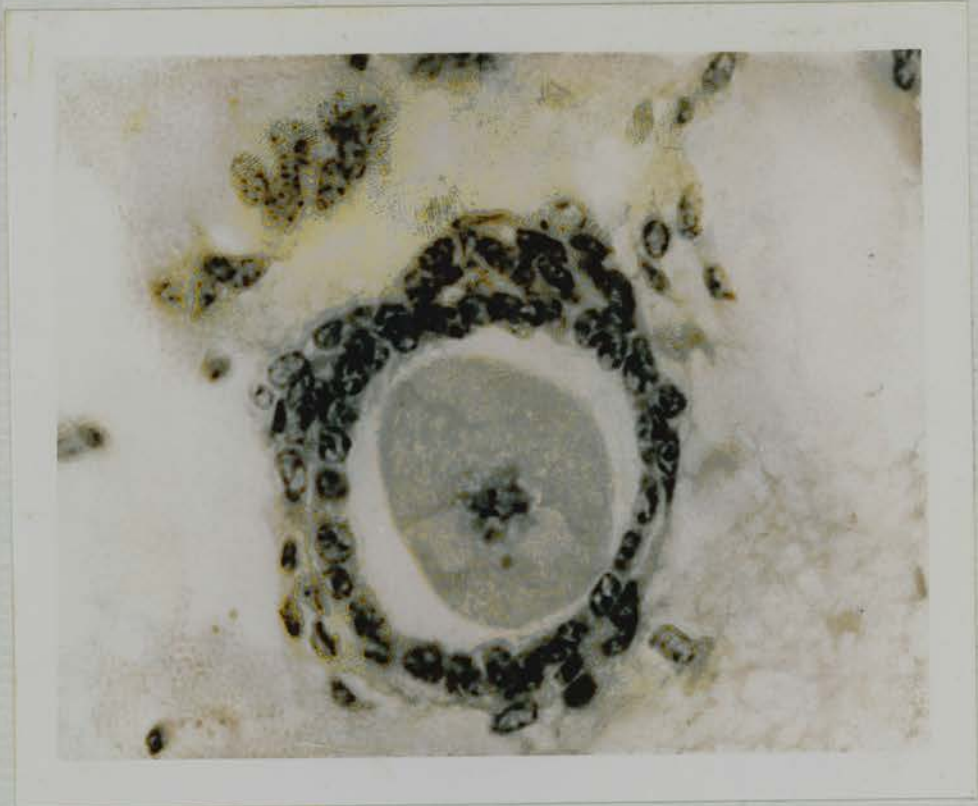


Figure 3.3. Section of 0 hour collagen gel showing oocyte contraction.

This degree of contraction away from the granulosa layers would result in the follicle being classed as intact but damaged (x 480).

histology to obtain information on the numbers, distribution and quality of follicles in the gels at the beginning of the culture period (0 hr). Groups of 4 gels from the same well were harvested at intervals during the culture period (see Figure 3.4.) so that an impression of the pattern of growth over a 14 day culture period would be gained. The results from 11 disaggregation experiments and more than 100 gels are reported. The same batch of serum was used for each experiment. Differential follicle counts using the above classification system were carried out on all gels.

Every section was counted in the 0 hour gels and in gels cultured for 6 or more days in which numbers were lower. Every third section was counted for gels from 1 to 5 days in culture. Follicles were counted only when the follicle section contained the nucleolus of the oocyte. Nucleolar diameters were measured in 22 follicles for Stage I, II and III follicles in 0 hr gels using an image shearing micromasurement system (Vickers Instruments, York, UK) under x100 oil immersion optics. These measurements were used to estimate a correction factor to compensate for overcounting resulting from the possibility of the nucleolar marker being present in more than one section. Correction factors were obtained using the equation (Abercrombie, 1946):

$$\frac{\text{Section thickness}}{\text{Section thickness} + \text{nucleolar diameter}}$$

Total numbers of follicles were calculated by multiplying the number counted by 3 (the sampling frequency) and the correction factor. As Stage V follicles were absent in the 0 hr gels, the Stage IV correction factor was used. Stage I (primordial) follicles were present in the gels but were not counted as they were difficult to classify accurately at this magnification. Free oocytes and disrupted follicle numbers were estimated using the Stage III correction factor.

The reliability of the differential count was assessed. Three gels were counted 4 times, with at least 3 days between recounts. One gel was counted by another operator.

Measurements were also made of follicle and oocyte diameters. Follicles from intact ovaries and isolated follicles from 0 h, 3, 6 and 9 days in culture were used. Stage II, III and IV follicles were measured, Stage II follicles were subjectively classified as small (IIa) or large (IIb) before being measured. Follicles were only measured when the nucleolus was present and there was no histological evidence of damage. Intact ovaries came from 10-day-old F1 mice and were fixed in Bouin's fluid and processed as the gels. Measurements were made using a computer assisted image analysis system (μ Magiscan, Joyce Loebel). The use of computer assisted image analysis is discussed in more detail in chapter 5. Between 15-51 follicles were measured for each stage.

Manipulation of follicle numbers

This experiment was designed to investigate the influence of the density of follicles within the gel on growth and development. Duplicate experiments were carried out. Follicles were isolated as before using 24 mice for each experiment. After concentration of the follicles in the centre of the watchglass, samples were pipetted from the central concentration into the wells of a Terasaki plate. The rotation and pipetting were repeated until 9 samples had been obtained, three samples each of 5 μ l, 10 μ l and 20 μ l. A tissue was used to remove excess fluid and collagen gel was added as before. Each group of three gels was transferred to the wells of a Linbro tissue-culture plate containing 2 ml of culture medium and cultured as above. After 6 days in culture the gels were harvested, fixed and processed for histology. Every section was

viewed and differential follicle counts using the above classification system were carried out on all gels.

Electron Microscopy

A disaggregation using the ovaries from 20 mice was carried out as above. The final suspension of follicles was pelleted in a small test tube and the pellet fixed in 1% glutaraldehyde for 1 hour followed by a further hour in 1% osmium tetroxide in 0.13 M Pipes buffer (pH 7.0). Dehydrated tissue was embedded in LR White resin (London Resin Company, London, UK) and thin sections cut with a Porter-Blum MT2 microtome using glass knives. Sections were mounted on single-hole type-1000 grids and stained with aqueous uranyl acetate followed by lead citrate.

Serum Bioassay

Donor and fetal calf serum (Flow Laboratories, Irvine) were assessed for gonadotrophic (FSH) activity. Forty 20 day old, CH₃ female mice, weighing between 9.75 and 10.20g were used. Each mouse was primed with hCG and received a total of 40 i.u of human Chorionic Gonadotrophin (Chorulon, Intervet Laboratories, Cambridge, UK), given by daily subcutaneous injection over three days. Eight groups of five mice were established and treated as shown in Table 3.1. Serum and FSH (NIADDK-oFSH-16, NIH, Bethesda, USA) were given by careful subcutaneous injection to avoid leakage. Injection volumes over 0.2 ml were split between two sites. All mice were killed on the fourth day and the ovaries dissected free of surrounding tissue and weighed in pairs. FSH standards were used for constructing a dose/response curve. The bioassay was repeated using outbred mice.

Table 3.1. Treatment groups for serum bioassay.

	<u>Dose per day</u>	<u>Total for 3 days</u>
<u>Group A</u> Donor calf serum	0.2 ml	0.6 ml
<u>Group B</u> Donor calf serum	0.8 ml	2.4 ml
<u>Group C</u> Fetal calf serum	0.2 ml	0.6 ml
<u>Group D</u> Fetal calf serum	0.8 ml	2.4 ml
<u>Group E</u> Saline	0.2 ml	0.6 ml
<u>Group F</u> FSH	0.1 i.u.	0.3 i.u.
<u>Group G</u> FSH	0.2 i.u.	0.6 i.u.
<u>Group H</u> FSH	0.6 i.u.	1.8 i.u.

RESULTS

Longitudinal study of follicle growth

Figure 3.4 shows the mean number of oocytes per gel over the course of the culture. The mean number at 0 hours was 184 ($n = 42$), the range was from 43 to 445 oocytes per gel. An average of around 200 follicles per gel had been aimed for. The number of oocytes dropped markedly after 6 days. No overall pattern is apparent in the number of free oocytes and follicle fragments over the culture period.

Figure 3.5 summarises the data of the follicle counts for individual gels over the culture period. The mean number of growing follicles at 0 hours was 115, range 21-248 ($n = 42$). The proportion of intact follicles with some histological evidence of damage ranged between 33-66% between Days 2 and 5. On days 6-8 the percentage of intact but damaged follicles decreased to 22, 28 and 17%, and then increased after Day 9 to 40%. By Day 14 64% of the follicles were judged intact but damaged.

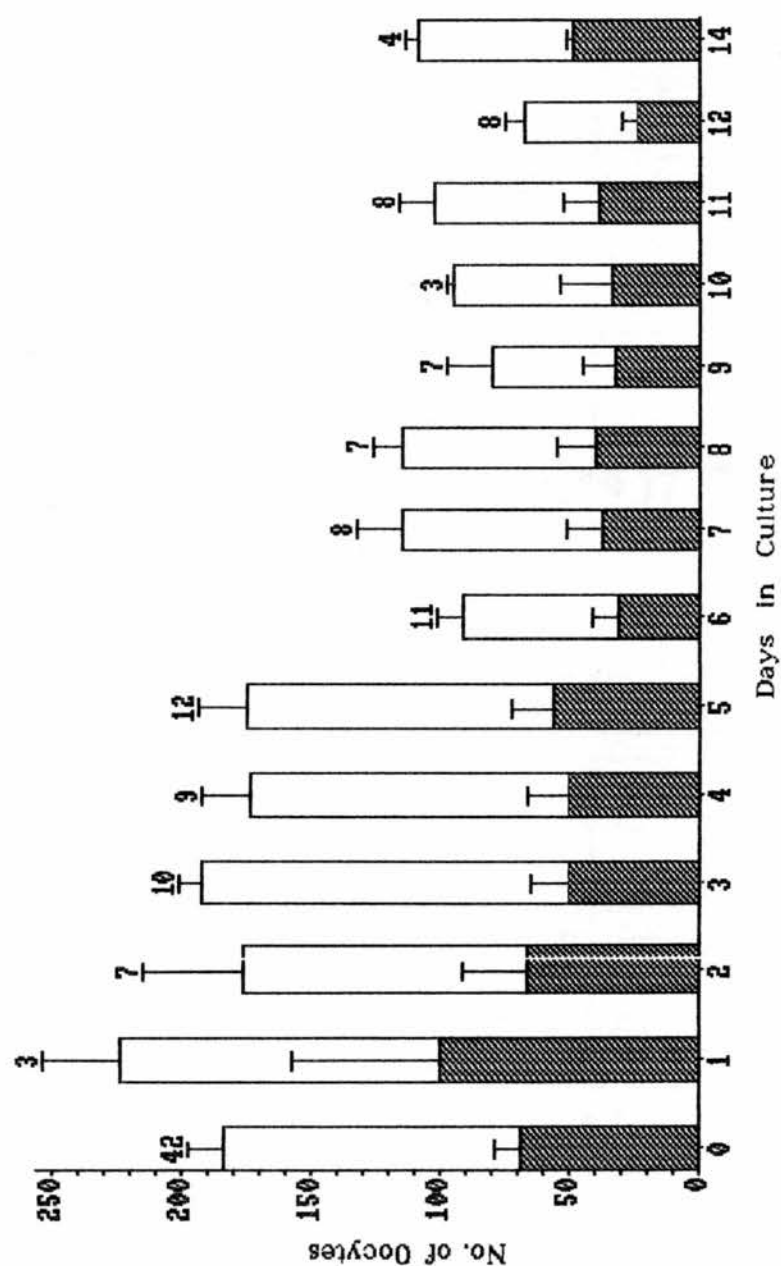


Figure 3.4. A representation of the mean number of oocytes per gel over 14 days in culture. The hatched bars represent oocytes in damaged follicles (including free oocytes) and unhatched bars represent oocytes in intact follicles. Values given are mean \pm s.e.m. The number of gels counted (n) is shown above the bars.

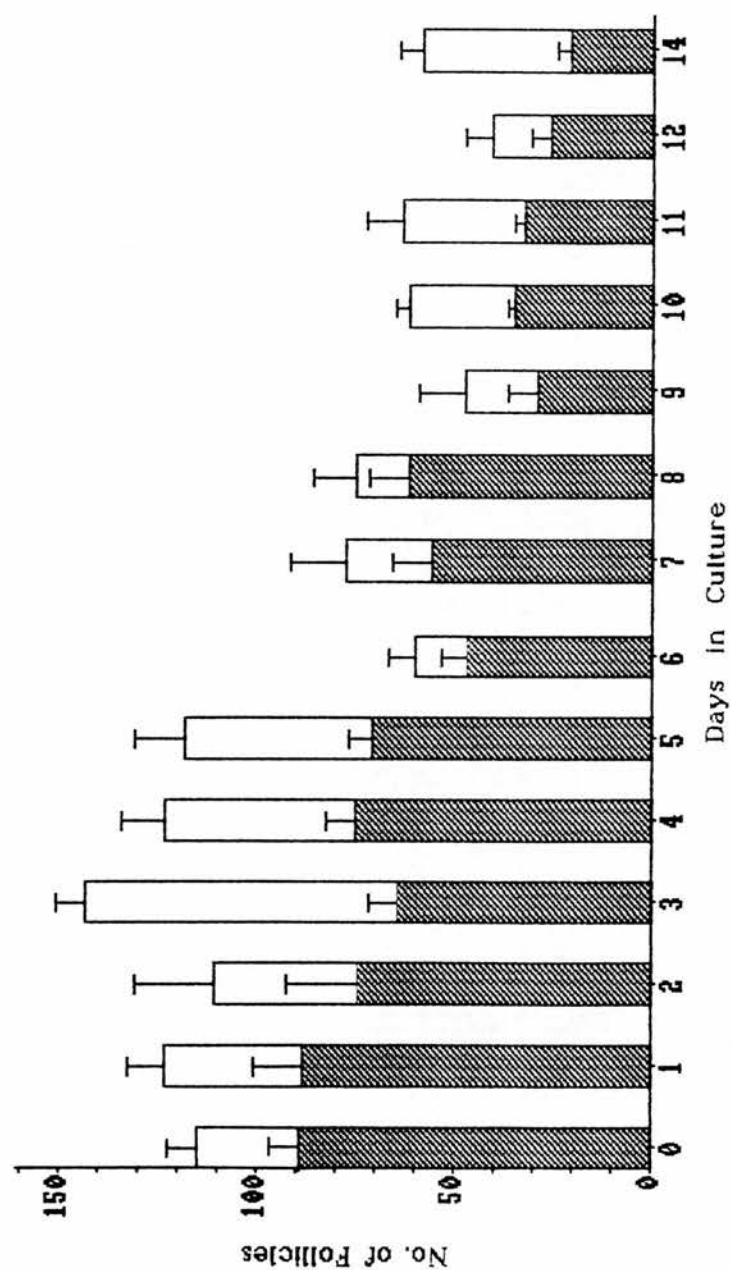


Figure 3.5. A representation of the total number of follicles (of all stages except primordial) per gel over 14 days in culture. The hatched bars represent morphologically normal follicles and the unhatched area represents intact follicles with evidence of damage. Values are given as mean \pm s.e.m.

The distribution of follicles of each stage (II-V) over the course of the culture period is shown in Figure 3.6. The most marked change is the increase in numbers of stage IV and stage V follicles. At the start of the culture period (0 hr) 43% of follicles were Stage II and 42% Stage III with only 5% being Stage IV. After 6 days in culture the distribution had altered with 36% of follicles being Stage II, 30% Stage III, 17% Stage IV and 10% Stage V (i.e with 4 or more layers). By 14 days in culture the distribution had shifted to only 16% of follicles being Stage II, 16% Stage III, 24% Stage IV. 44% were Stage V follicles. Stage V follicles had 4 or more layers yet remained preantral. Antral follicles were not identified at any time in culture and occasional spaces between granulosa cells were attributed to histological artefacts.

At 0 hour about 22% of the intact follicles showed histological evidence of damage. Between Days 2 to 5 there was a gradual increase in the number of larger follicles but the proportion of unhealthy follicles also increased. Between Days 6 to 8 the proportion of damaged follicles fell, 80% of follicles of all stages were free of histological evidence of damage by Day 8. The proportion of unhealthy follicles then increased and was 64% on Day 14.

Observation of the gels by phase contrast microscopy indicated that the follicles retained their spheroidal form, with the oocyte maintaining a central position (Figure 3.7). Numerous processes were observed growing outwards from the granulosa cells. As the cultures progressed the collagen gels tended to shrink making viewing more difficult. Figure 3.8 shows a section of a 0 hour collagen gel: clumps of primordial follicles, intact follicles with 1 and 2 granulosa layers and damaged follicles can be seen. Figures 3.9 - 3.11 show enlarged views of individual follicles in control gels. After several days in culture follicles maintained an appearance

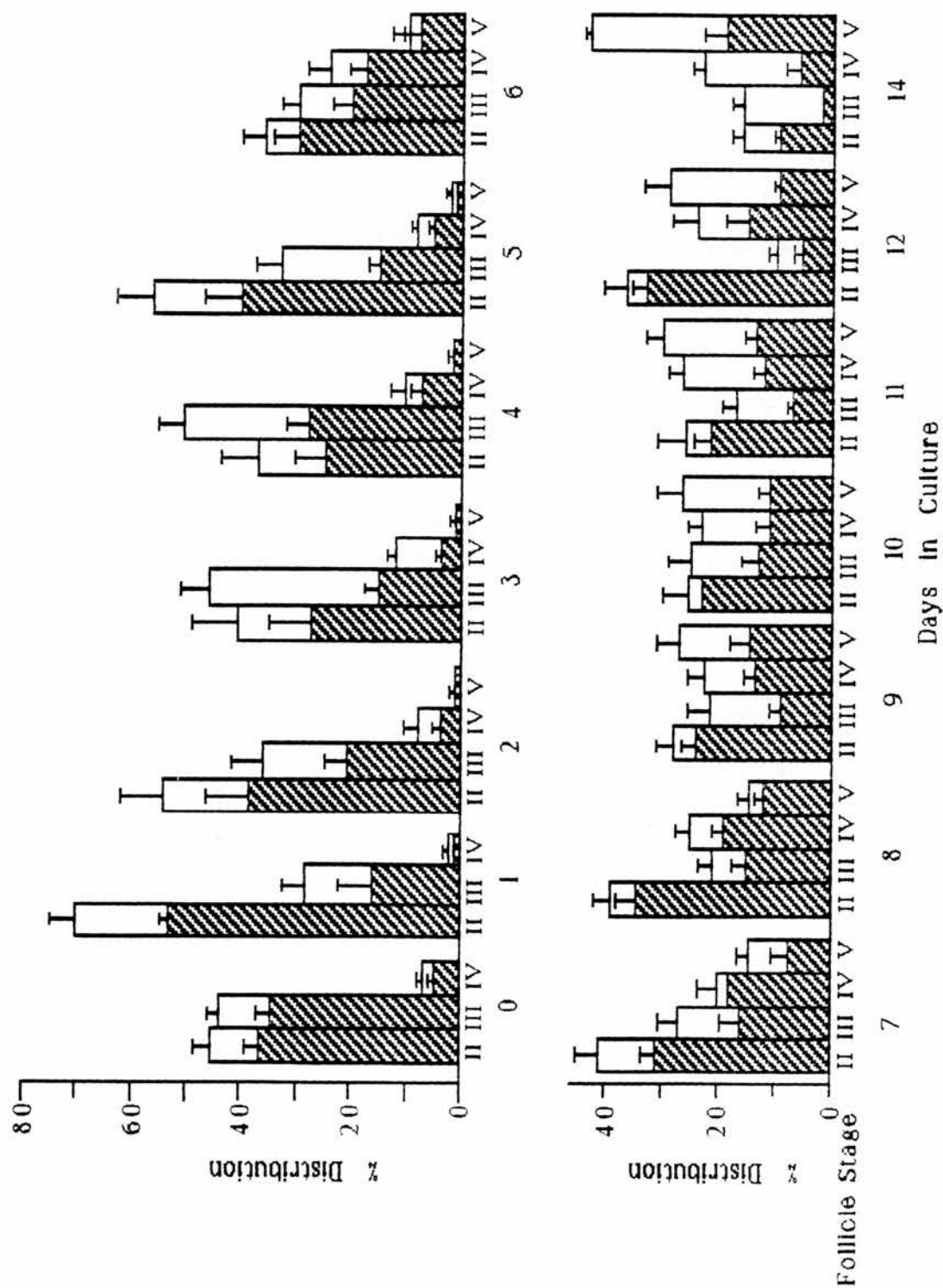


Figure 3.6. Distribution of follicle stages (II-IV) as a percentage of the total number of intact follicles per gel from 0-14 days in culture. Hatched area represents intact follicles with no histological evidence of degeneration. Values are given as mean \pm s.e.m.



Figure 3.7. Follicles in collagen gel during the culture period. The follicles maintain their structure and show cellular outgrowths (x 150).

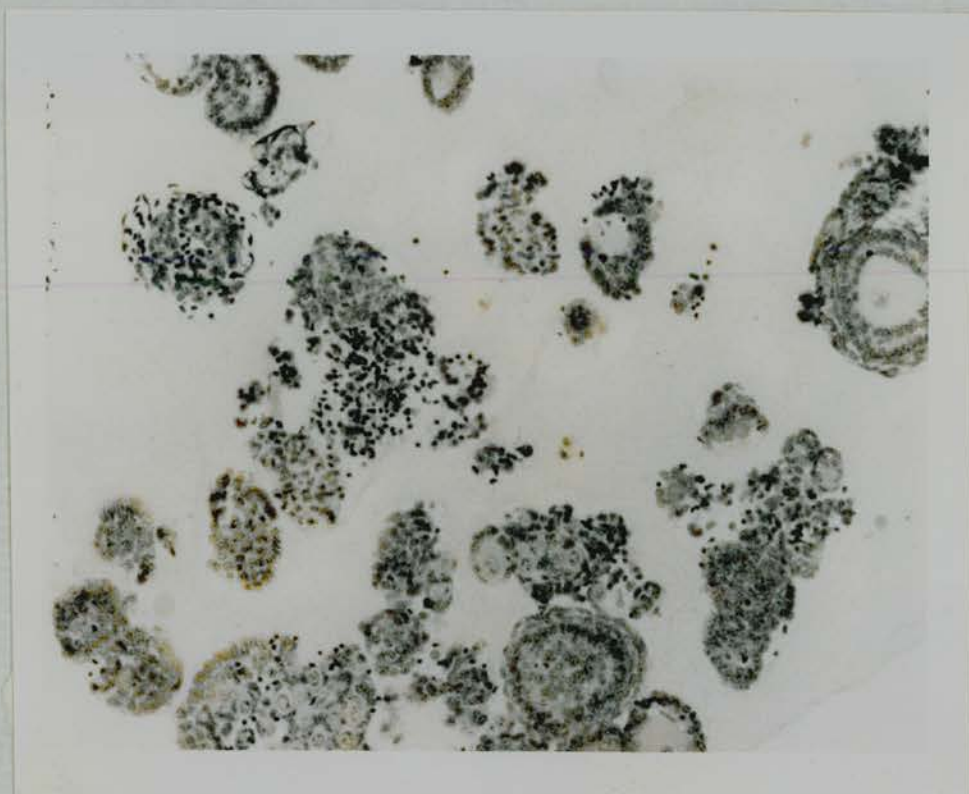


Figure 3.8. Section of 0 hour collagen gel showing 1 and 2 layered follicles, clumps of primordial follicles and some damaged follicles. Pyknosis is evident in cell clumps (x 120).



Figure 3.9. Section of 0 hour collagen gel showing an isolated, small stage II follicle (x 480).



Figure 3.10. Section of 0 hour collagen gel showing stage III follicles with second layer of granulosa cells only partially formed (x 480).



Figure 3.11 Section of 0 hour collagen gel showing a stage IV follicle with three granulosa cell layers (x 480).

very similar to that in the intact ovary (Figures 3.12 & 3.13). The main difference between the intact ovary and cultured follicles was the presence of multilayered follicles after 6 days in culture. The ovary from the 10-day-old mouse (Figure 3.13) contained no Stage V follicles but these represented 27% of the intact follicles by Day 12 in culture. Although no counts were made of primordial follicles, they were present at all times and had a normal histological appearance.

The oocyte and follicle increased in size at each stage in culture for all groups (Figure 3.14). There were no significant differences in oocyte and follicular diameter with time in culture compared with the intact ovary. The subjective classification of 1 layer (Stage II) follicles into small and large appeared to produce consistent results. Some follicles with one layer of granulosa cells (Stage IIb) had oocytes as large as those in Stage III follicles. There were no significant differences in oocyte or follicle diameter with time in culture compared with the intact ovary. This indicated that follicular growth was proceeding with a degree of normality.

The data from follicle recounts are shown in Figures 3.15 - 3.19. Figure 3.15 shows the number of follicles per gel for four counts per gel of three gels. The hatched area represents histologically normal follicles, the clear area represents follicles with evidence of damage. The number of follicles ranged from 27-32 for a gel after 10 days in culture, 111-148 for a 3-day gel and 97-99 for a 0 hour control gel. Reliability was high although the range in the 3-day gel (every third section counted) was greater than in the control and 10-day gels (every section counted). Figure 3.16 summarises the data for the recount of another gel by a second operator. The assessment of intact follicle quality was not checked. Figure 3.17 summarises the distribution of follicles of each stage for 4 counts each of a control and 10-day cultured gel.

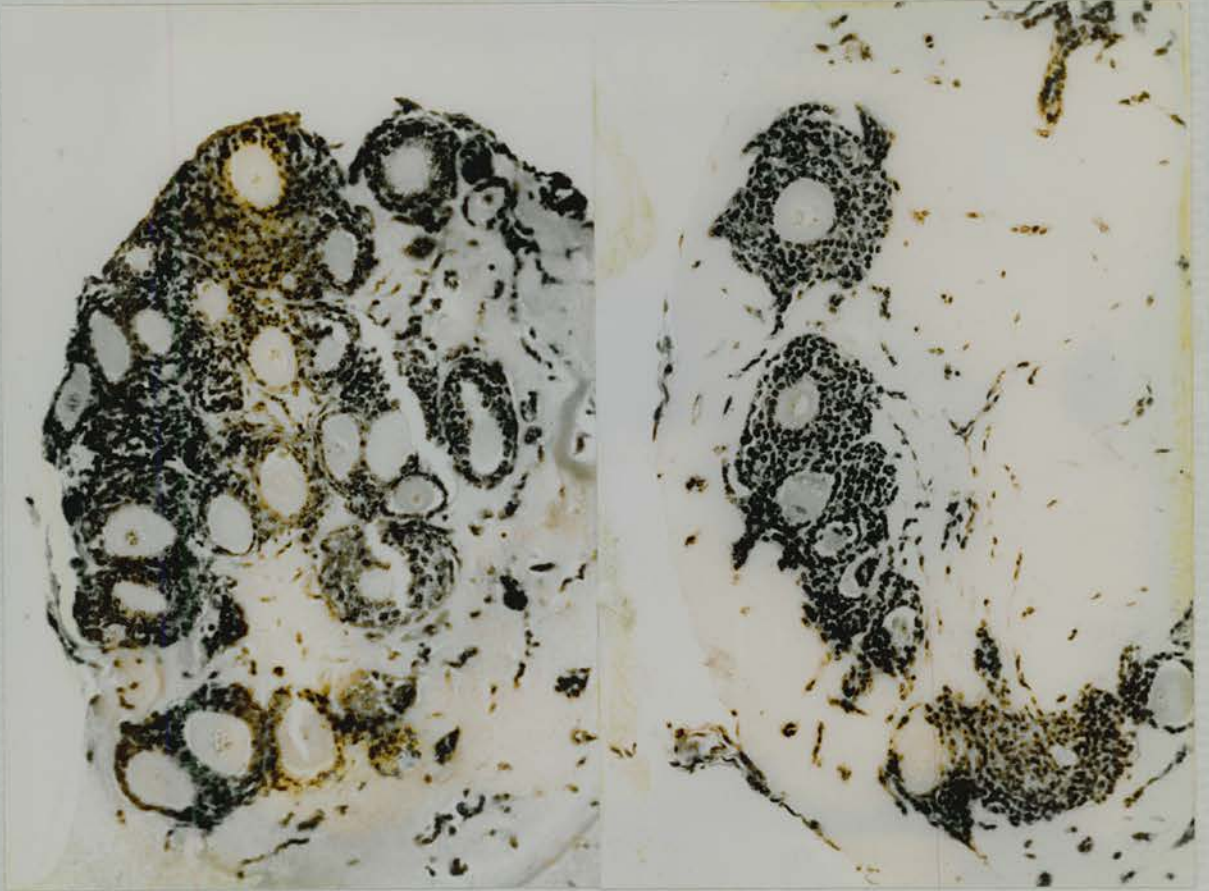


Figure 3.12. Histological sections of a gel containing follicles after 6 days in culture. Unilaminar and multilaminar follicles can be seen. Some of the multilaminar follicles are showing signs of degeneration (x150).



Figure 3.13. Section of an intact ovary from a 10-day-old F₁ hybrid mouse. No multilaminar follicles or theca are found in the ovaries at this age (x 150).

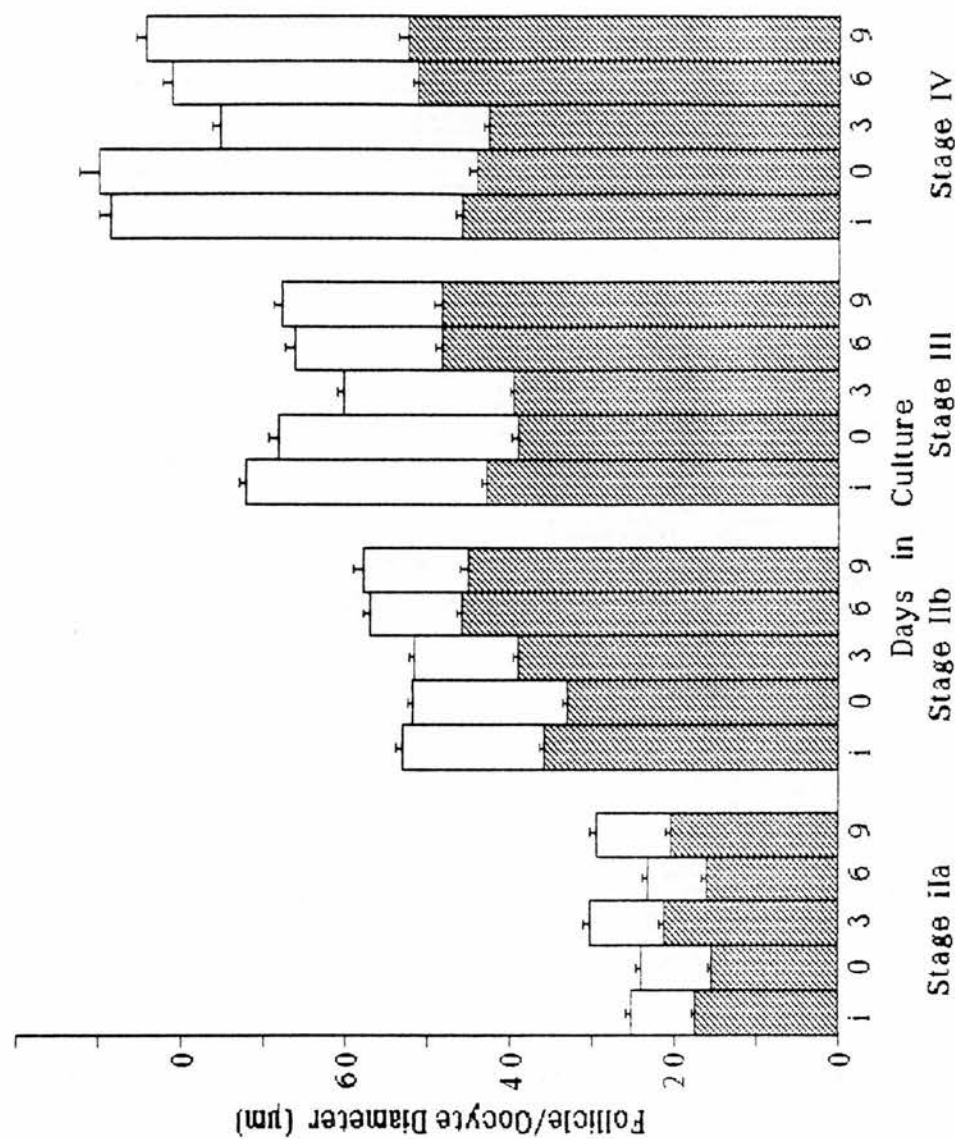


Figure 3.14. Mean follicle and oocyte (hatched area) diameters at four follicle stages in

Intact ovaries (i) and isolated follicles up to 9 days in culture. Values are

given as mean \pm s.e.m., n ranged from 15-51 follicles.

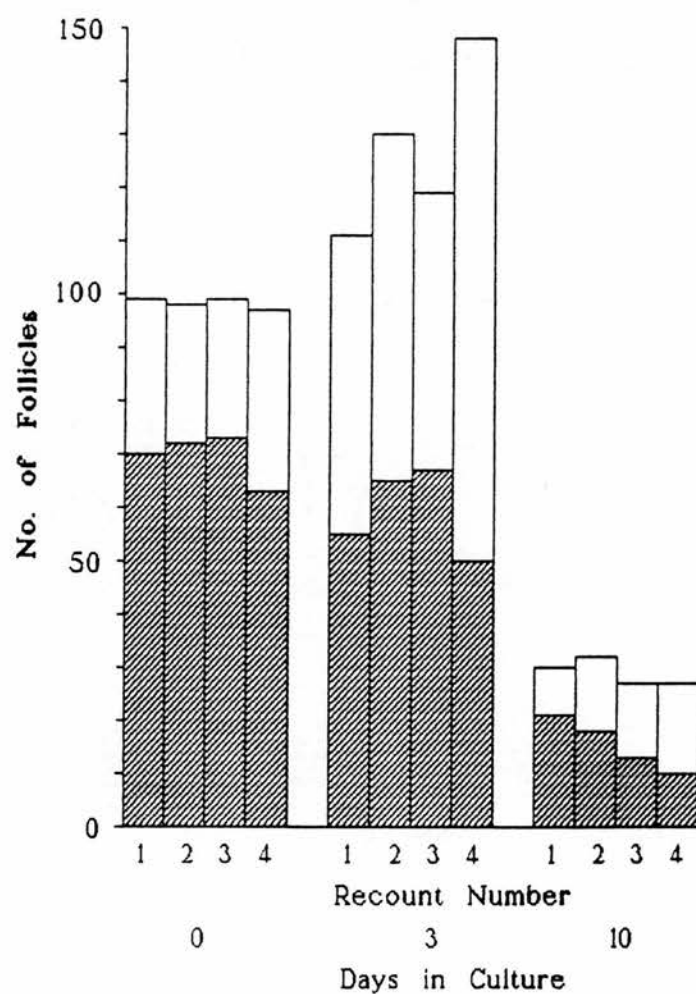


Figure 3.15. A representation of the total number of intact follicles per gel for 4 recounts of each gel. The hatched area represents follicles with no histological evidence of degeneration.

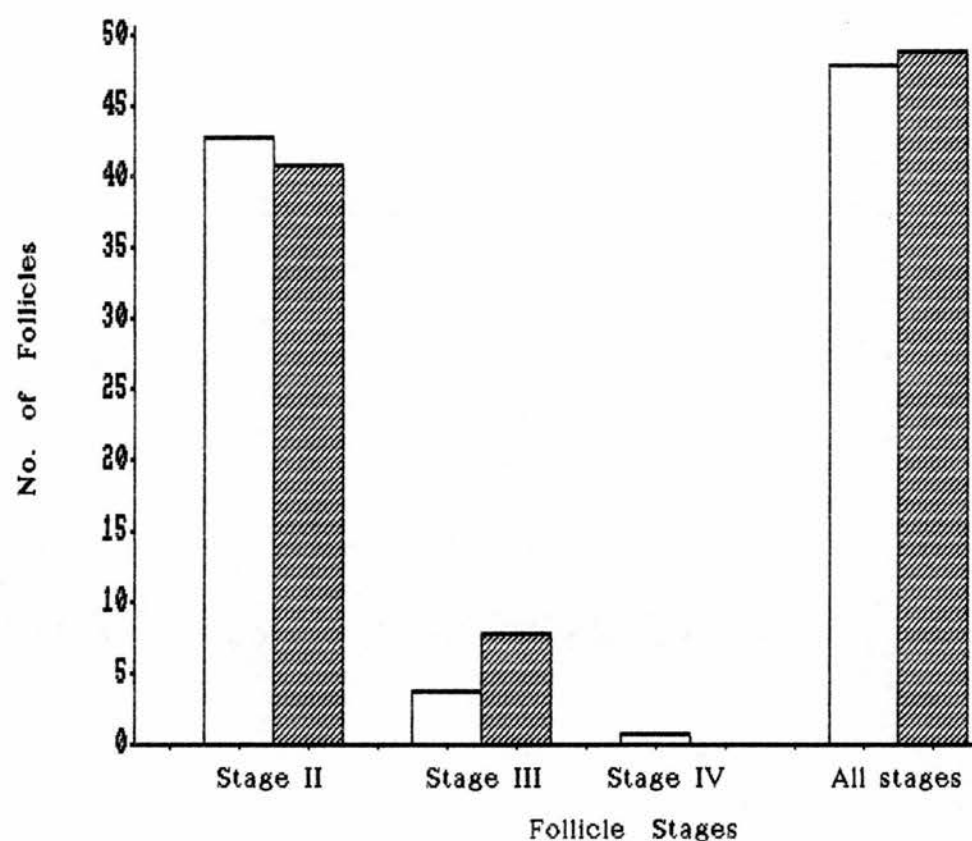


Figure 3.16. Distribution of follicle stages showing a recount by a second operator (unhatched bars) and original count (hatched bars) of a single collagen gel.

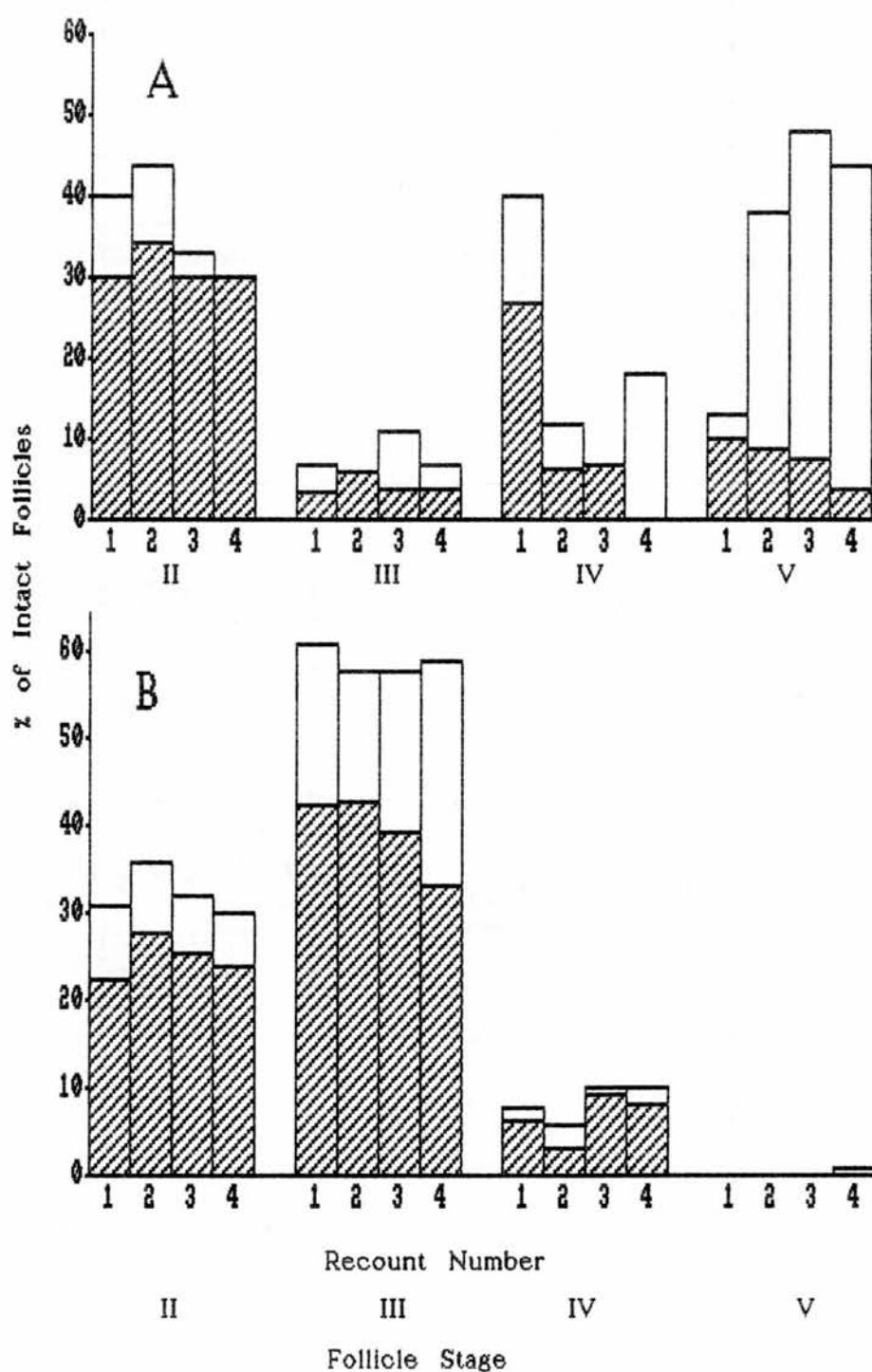


Figure 3.17. Distribution of follicle stages (II-V) as a percentage of the total number of intact follicles per gel for recounts (1-4) of a cultured (A) and control (B) gel. The hatched area represents the percentage of intact follicles with no histological evidence of degeneration. Gel A was cultured for 10 days.

Electron microscopy of freshly isolated follicles indicated that intact follicles had a normal or near normal EM appearance (Figure 3.18). However basement membrane damage was identified.

Manipulation of follicle numbers

Data were only available on gels containing 5 and 10 μ l of follicle suspension. The 20 μ l samples were lost during histological processing. The results are summarised in Table 3.2. As sample size was small (4 gels per group) and one group had been lost statistical analysis was not carried out. The most interesting feature is the difference in the number of Stage V follicles. Both groups had about the same proportion of Stage IV follicles (5 μ l group 21%, 10 μ l 19%) but the low follicle density gels had an average of only 2% Stage V follicles compared with 8% in the higher density group. The figure of 8% for the higher density group compares favourably with the 10% Stage V follicles reported on Day 6 of the main study.

Serum Bioassay

The ovarian weights for each treatment group are given in Table 3.3. The CH3 mice proved to be insensitive to the levels of FSH in the standards and a slight increase in ovarian weight was only seen in the highest dose (1.8 i.u.). A dose response curve was not demonstrated. The sera tested did not show any marked gonadotrophic activity. The largest uterine weight was produced by the 0.6 ml fetal calf serum treatment. The second bioassay using outbred mice similarly indicated undetectable gonadotrophic activity.

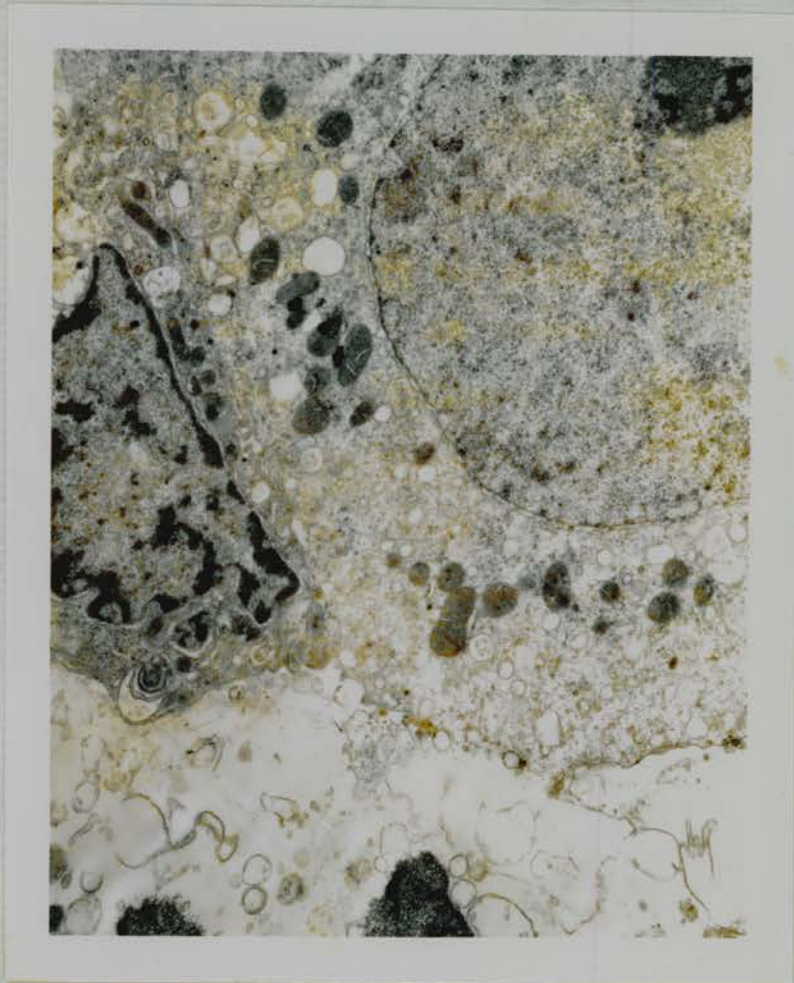


Figure 3.18. Section of a primordial follicle after enzymatic isolation. The oocyte has sparse short processes, no zona pellucida or delicate basement membrane can be seen (x 5000).

Table 3.2. Differential follicle counts on gels cultured for 6 days
containing 5 or 10 μ l of follicle suspension.

	<u>Volume of follicles in collagen gel</u>	
	<u>5 μl</u>	<u>10 μl</u>
Total No. Oocytes	39 \pm 1.0	163 \pm 1.05
Intact Follicles	28 \pm 9.1 (21)	101 \pm 9.8 (83)
Stage (% of intact follicles)		
II	30 \pm 2.9 (25)	39 \pm 5.7 (34)
III	46 \pm 3.0 (31)	32 \pm 2.9 (26)
IV	21 \pm 2.2 (19)	19 \pm 2.3 (15)
V	2 \pm 1.1 (2)	8 \pm 0.4 (7)

Values are given as the mean \pm s.e.m for 4 gels. The numbers in brackets represent intact follicles without any histological evidence of degeneration.

Table 3.3 Mean ovarian weight for CH3 mice after serum or FSH treatment.

<u>Treatment</u>		<u>Ovarian Weight (mg)</u>
Donor calf serum	0.6 ml	3.74 \pm 0.4
	2.4 ml	4.82 \pm 0.3
Fetal calf serum	0.6 ml	5.28 \pm 0.4
	2.4 ml	3.28 \pm 0.7
Saline	0.6 ml	4.08 \pm 0.7
FSH	0.3 i.u.	4.78 \pm 0.6
	0.6 i.u.	4.44 \pm 0.1
	1.8 i.u.	5.42 \pm 0.7

(For each treatment n = 5, values are mean \pm s.e.m.)

DISCUSSION

The technique developed permits mouse ovarian follicles to be isolated and grown *in vitro* for up to 2 weeks. Using this dissociation procedure large numbers of isolated follicles can rapidly be produced. On average it takes about 3 hours to isolate the follicles and place them in culture. Histological and EM assessments indicate that collagenase treatment does not cause major or irreversible changes to the follicular unit. Roy and Greenwald (1985) have developed a similar dissociation method using collagenase and pronase to isolate the follicles of hamsters but have only used follicle suspensions in short term incubations to study hormone production and receptors (Roy & Greenwald 1987). The 10-day-old mice were chosen because their ovaries rarely contain stage V follicles (follicles with four or more granulosa layers). The multilaminar follicles seen after 6 days in culture must therefore have grown *in vitro* from small or even primordial follicles. The follicles of the 10-day old mice used did not have a theca and the system failed to support thecal or antral differentiation *in vitro*. However the culture system was the first reported to support the *in vitro* growth of small follicles to the multilaminar stage. Measurements of oocyte and follicle diameters indicate that the follicles were developing as a complete unit, similar to the pattern seen *in vivo*. The major aim of the study, to demonstrate that the collagen culture system can support growth of small follicles *in vitro* has been achieved.

In previous studies (reviewed in chapter 2) follicles could only be cultured for a short time before the 3-dimensional integrity of the follicular unit was disrupted. In this study the collagen gel provides a more physiological matrix maintaining the 3-dimensional shape and relationships of the follicles without evidence of the limitation of

diffusion of gases or metabolites seen in organ cultures. The collagen gel system also provides other advantages, i.e. a convenient method for handling follicles during processing for histology and a convenient vehicle for handling the follicles during transplantation under the kidney capsule, as reported in the next chapter.

The system has been shown to be capable of supporting follicle development up to the large multilaminar stage. These follicles are clearly growing from unilaminar follicles. It remains to be determined if this growth is due to progression of small but growing unilaminar follicles or to recruitment from the pool of non-growing primordial follicles. Approaches to investigating recruitment of primordial follicle *in vitro* are being developed. A paper tissue is used to remove excess fluid before adding the collagen gel solution and some primordial follicles are probably being lost at this stage. Improvements in the isolation procedure may improve the quantity and quality of the isolated follicles.

The relatively high proportion of unhealthy follicles during the first few days in culture suggests that this is a result of damaged follicles being put into culture. These follicles may have been damaged by either the enzymes used to dissociate the ovaries or by the mechanical trauma of repeated pipetting. The decline in the proportion of unhealthy follicles at all stages after 6 days suggests that follicle growth is proceeding normally. Small follicles were usually indistinguishable from their *in vivo* counterparts but many of the large multilaminar follicles showed evidence of degeneration, mainly pycnotic cells, and neither a distinct theca nor antrum development was observed in any of the cultured follicles. However a few follicles occasionally had some spindle-shaped cells around parts of the outer granulosa layer. This finding was not consistent and at best represented a proto-theca (an example is shown in Figure 6.1). It would

appear that the multilaminar follicles in culture had reached a stage where they might require additional support for further growth. The work of Qvist et al. (1990) and of Dr P. Nayudu (personal communication) on dissected preantral follicles from more mature mice indicates that FSH is required for antrum formation and to support thecal differentiation.

Medium supplemented with donor calf serum was chosen to represent basal conditions as it contains little gonadotrophin or sex steroids but can be expected to contain non-specific growth factors. The results of the bioassay confirm that the medium used had little gonadotrophic activity. Follicular development was blocked at multilaminar stages, neither theca or antra were formed *in vitro*. The failure of theca and antra to form in these conditions could be due to either an intrinsic inability of the follicles to develop further, (because of damage during the dissociation or abnormal development *in vitro*) or as a result of limitations of the culture system. Failure to produce thecal differentiation is a major limitation of the method. The rapid attachment and spreading of the follicles when cultured directly on a plastic substrate may be evidence of damage to the basal lamina and as discussed in the previous chapter ECM may have an important inductive role in mesenchymal-epithelial interactions. The results of *in vivo* transfer (Chapter 4) of *in vitro* culture follicles shows that the cultured follicles have the potential for thecal development and antrum formation. The results indicated that there is no inherent inability of the follicles to develop beyond the multilaminar stages and that the limiting factor to further development appears to be the culture conditions, perhaps the lack of an essential hormone or of growth factors..

As the ovarian follicle progresses from the primordial to Graafian stage *in vivo* it may experience a sequence of local and systemic hormonal environments that are essential for progression to the next stage but

which the current system has not yet duplicated *in vitro*. Additionally features of the intact ovary, for example ovarian blood vessels, nerves or the surface epithelium may influence some stages of follicular development. To identify the essential factors for growth in an *in vitro* system can be a difficult and lengthy task, particularly when the coordinated development of several cell types is required. Later chapters deal with investigations of hormonal influences and initial steps in developing a defined medium capable of supporting follicle growth.

The follicle suspension contains a variety of cells - follicle units, damaged follicles and isolated granulosa cells, stromal cells, ovarian surface epithelium, and other cell types. It would be useful to separate follicles into stages and look for stage-specific effects or secretions. The last chapter discussed initial attempts to produce a purified follicle suspension and to separate the follicle stages. This can be done using calibrated pipettes (Roy & Greenwald, 1985) but a method which would allow large numbers to be conveniently handled is required. Greenwald and Moor (1989) have developed a method of producing large numbers of primordial follicles from the pig ovary. They incubated slices of pig ovary for 2 hours in 0.025% collagenase, used a discontinuous Percoll gradient to remove contaminating somatic cells and harvested between 100-1500 primordial follicles. For large scale recovery they recommend flow cytometry. A recent study reports the use of Percoll gradients and micromeshes to separate rapidly large numbers of collagenase-isolated rat follicles (Conway et al., 1990).

The system developed has potential for investigating many aspects of the development of small ovarian follicles. The histological approach allows gross morphological changes such as an increase in granulosa cell layers to be identified. However for more subtle changes in follicle function

other techniques are necessary. One approach used in later chapters is the application of autoradiography to investigate changes in protein metabolism and the effects on the granulosa and oocyte compartments. Separation of the follicle stages and gel electrophoresis has potential for identifying stage-specific markers. The techniques need much more development and validation with particular attention to establishing a defined medium, to separating the different follicle stages and cell types, and identifying more subtle markers of follicle differentiation. With development the system could be used to investigate the factors controlling two major transition points in follicle development: the recruitment of quiescent primordial follicles into the growing pool and antrum formation.

Since this work was completed a number of papers using different culture systems have appeared. There is evidence that without a 3-dimensional matrix the purine, hypoxanthine, can maintain oocyte-granulosa cell complexes, isolated from 10-11 day old mice and cultured for 12 days in monolayer conditions (Eppig & Downs, 1987). It would seem that hypoxanthine is maintaining the relationship between the oocyte and granulosa cells because some 50-70% acquire competence to undergo germinal vesicle breakdown. Daniel et al. (1989) have used a similar collagenase procedure to obtain preantral rat follicles. The follicles were then cultured on a plastic substrate in medium supplemented with hypoxanthine. Oocytes grew and acquired meiotic competence during culture for 20 days and increased production of oestrogens, androgens and progestins was recorded. Between 25-33% supported sperm penetration after *in vitro* insemination but a high proportion showed abnormal fertilization. The collagen gel approach is aiming for a more physiological situation where the follicle unit is intact but the effect of hypoxanthine on follicles in collagen gel culture needs to be assessed.

An abstract by Maresh & Dunbar (1987) briefly mentions culturing isolated rabbit follicles on a variety of substrates including poly-D-lysine, collagen, laminin and EHS basement membrane type biomatrix. The EHS matrix is reported to maintain the normal spherical morphology. The authors also report the use of two-dimensional polyacrylamide gel electrophoresis to identify follicle secreted glycoproteins. A complete report of the work has not yet been published. The addition of other ECM constituents to the basic collagen gel and the use of a commercially produced, more complete ECM such as Matrigel, are planned developments of the system.

Most recently antrum formation *in vitro* has been demonstrated by Qvist et al. (1990). Clumps of small follicles were isolated with adhering stroma by dissection with fine needles. BALBc mice were used at 5-8, 12 and 16 days of age. Follicles of stage I-III were obtained and cultured on a hydrophobic membrane to prevent adhesion. The medium was supplemented with FSH, LH and 50% post-menopausal serum. Follicles from 5-day-old mice did not progress beyond 2 layers of granulosa cells but some follicles from 8-day-old mice developed to stage V. Follicles from 12-day-old mice developed to the large, multilaminar stage but with no antrum. These results correspond with the those reported in the present study using 10-day-old mice. Only follicles from 16-day-old mice formed an antrum *in vitro*. Early theca was present when the follicles went into culture but thecal differentiation *in vitro* was incomplete, and even the most advanced preovulatory oocyte had not resumed meiosis. Oestradiol production was undetectable in cultures of follicles from 5 and 8-day-olds. Spent culture medium from follicles of the 12-day-old mice contained increasing concentrations of oestradiol, peaking at 13.5 nM after 11 days in culture then decreasing. A similar increase was noted for the 16-day-old mice with

a peak of 22 nM at 14 days. The authors concluded that follicular growth *in vitro* was dependent on adequate levels of FSH. Addition of FSH to the collagen gel system (Chapter 5) did not stimulate thecal or antral development in follicles obtained from 10-day-old mice. The block on development of follicles at the multilaminar stage V for follicles from 8-day-old mice is similar to that found in the collagen gel system, as is the lack of full thecal development *in vitro*. The difference between the *in vitro* growth potential of follicles from 8 and 12-day old mice suggests that some *in vivo* priming signal is involved. However the use of 50% post-menopausal serum also limits the usefulness of the approach for investigation of factors which modulate follicle growth.

CHAPTER 4

A morphological study of cultured preantral ovarian follicles
after transplantation under the kidney capsule of ovariectomized hosts.

Introduction

In vitro techniques are useful tools for investigating many aspects of cell growth, differentiation and function. They enable the researcher to modify and control the environment of the cell to a degree that could not be achieved *in vivo*. However cells in culture may not express the full range of the differentiated features and function seen *in vivo*. Cells in culture often lose their histotypic patterns and the number and nature of intracellular connections and communication structures such as gap junctions may change. Epithelial cells in particular may lose their shape, hormone responsiveness and differentiated functions. It is necessary to identify if the lack of differentiated function *in vitro* is due to irreversible changes in the cells or if they have retained their differentiated identity and function but do not express it due to limitation of the culture system.

A method for isolating and culturing preantral follicles was developed, the follicles grew *in vitro* but development was blocked at multilaminar stages with neither theca nor antra formation occurring *in vitro*. These isolated follicles may be abnormal and incapable of further development due to the conditions in which they were isolated and cultured or alternatively development is limited due to the absence of critical signals for differentiation within the culture system. This chapter reports the results from a method designed to determine if these follicle were capable of further development when transferred *in vivo*. Follicles were cultured in collagen gel and then transplanted under the kidney capsule of ovariectomized hosts to investigate their developmental potential. The combination of *in vitro* and *in vivo* environments represents a novel approach to the study of folliculogenesis.

However the culture and *in vivo* transfer approach adopted has been utilised with other reproductive epithelia. Cooke et al. (1986) investigated the ability of vaginal and uterine epithelial cells to re-express their normal morphology and hormonal responsiveness after cell culture. Vaginal and uterine epithelia were grown in a collagen gel matrix with serum-free medium for 7-10 days. Cell proliferation occurred but was not oestrogen-dependent as *in vivo*. Vaginal epithelium did not keratinise or stratify *in vitro*. When the epithelia were combined with their respective stroma and transferred under the kidney capsule the epithelial cells proliferated and cycled appropriately in response to oestrogen and progesterone. Epithelia-only transplants did not survive *in vivo*. Mouse mammary epithelial cells grown in monolayers have been shown to regain some differentiated features after transfer to gland-free mammary fat pads *in vivo* (Daniel & DeOme, 1965).

Materials & Methods

Animals

The mice used in this study were F₁ females aged 8-11 days post partum and derived from matings between CBA/Ca males and C57Bl/6 females. Adult F₁ hybrid females were used as hosts for transplants. The mice were provided with a pelleted diet and water *ad libitum* and housed under a light/dark cycle of 14 hours light starting at 0700 GMT. Room temperature was maintained at 21°C. Hosts were bilaterally ovariectomized at six weeks of age and allowed to recover for at least 3 weeks before use. All operations were carried out under intraperitoneal tribromoethane anaesthesia (6.03g/kg body weight).

Follicle culture

Follicles were isolated and cultured in a collagen gel matrix as described in chapter 3. All steps were carried out using ice-cold preparation medium. The bisected ovaries were incubated with gentle rotation (1 Hz) at 37°C in a 10 ml test-tube containing Hepes-buffered medium 199 (Gibco) with 1.5 mg/ml collagenase (Sigma type I) and 40 units/ml DNase I (Sigma) for 30 minutes. Collagenase was removed by gentle centrifugation and the follicles freed by repeated pipetting. The isolated follicles were pelleted by centrifugation, the supernatant removed and the follicle pellet resuspended in 3 ml of medium in a watchglass. The follicles were concentrated in the centre of the watchglass by gentle rotation and samples drawn off from this area of concentrated follicles using a 20 µl Gilson pipette and placed in the wells of a Terasaki plate (Flow Laboratories). A paper tissue was used to draw off excess fluid and more follicle suspension added to produce a higher number of follicles in each well. The follicles were set in collagen gel derived from rats tails as before. The gels were cultured in the wells of a Linbro tissue culture plate (Flow Laboratories) containing 2 ml of bicarbonate-buffered medium 199 (Gibco) supplemented with gentamicin and amphotericin B and 10% donor calf serum (Flow Laboratories). The gels were incubated at 37°C with 5% CO₂ in a humidified incubator. The medium was changed at 24 hr and 72 hr.

Morphology of transplanted follicles

Experiments were designed to investigate the growth potential of cultured follicles after transplantation *in vivo*. From two disaggregations using 26 and 25 animals a total of 22 gels were obtained. Gels were cultured for 5 days and then a single gel transferred under the kidney

capsule of each host animal. A dorsal incision was made over the kidney and the kidney was exteriorised. To immobilise the kidney and prevent it being drawn back into the body cavity a small U-shaped glass rod was placed under the kidney. Using a dissecting microscope and fine watchmaker forceps the kidney capsule was lifted and a small tear made, the tips of another pair of forceps were inserted into the tear and used to ease the capsule free of the kidney. This created a small space into which the collagen gel was placed. Once under the capsule the gel was gently manipulated away from the site of the tear to prevent it falling out. The gels had contracted in culture and were relatively robust. They could be handled with watchmaker forceps if care was taken not to disrupt the matrix. The kidney was replaced and the body wall closed with suture.

Animals were sacrificed between 2-21 days so that an impression of the pattern of growth over this period would be gained. Vaginal smears were taken before and after the collagen gels had been inserted. Vaginal smears were obtained by lavage using a flame polished Pasteur pipette. The smear was allowed to dry and stained with Geimsa and examined without coverslip or mountant to determine the stage of the cycle. The numbers of leucocytes, nucleated epithelial cells, cornified epithelial cells and the amount of mucin in the smears were estimated and graded as +, ++ or +++. The smears were interpreted on the basis of the predominant cell types present. At the termination of the experiment hosts were killed by cervical dislocation and the kidneys were removed. Uteri were removed from all animals and weights were obtained. Each animal was examined at autopsy to ensure that no ovarian fragments had remained after ovariectomy. The grafts were dissected and fixed overnight in aqueous Bouin's fluid, embedded in paraffin wax and sectioned at 7 μ m. Sections were stained with haematoxylin and eosin and mounted with DPX. Slides were examined for

presence of follicles with a differentiated theca and for the presence of Graafian follicles.

Six ovariectomized animals received tissue free, gel-only transplants. The animals were killed after 12 days and the uteri removed and weighed, to serve as controls. One uterine horn from a control and one from an experimental animal were fixed in aqueous Bouin's fluid and processed for histology (Appendix 1). Sections were cut at 7 μ m, stained with haematoxylin and eosin and mounted with DPX.

Observations on blood vessels and theca

Isolated follicles obtained from 25 animals were placed into 7 gels and after 5 days in culture the gels were transplanted into 7 hosts. One animal a day was sacrificed between 3-9 days after transplantation. Grafts were removed and fixed in formaldehyde for 1 hour then in formol calcium overnight, embedded in paraffin wax and sectioned at 7 μ m (processing details in Appendix 1). Alternate ribbons of sections were stained with either haematoxylin and eosin or with horseradish peroxidase (HRP) to identify blood vessels. Eosin is not an effective stain for discrimination of red blood cells (RBC) in dense tissues and HRP visualised by oxidation of 3,3'-diaminobenzidine tetrahydrochloride (DAB) was used to identify the presence of RBCs. When oxidised DAB produces a brown, alcohol-insoluble end product. Sections were counterstained with methyl green pyronin. All sections were examined microscopically: the H&E group was examined for the presence of large follicles and theca cells and the HRP group for associated blood vessels.

Observations of oocyte sperm binding capability:

Follicles were isolated from the ovaries of 10 mice and placed in 3 gels

and cultured as above for 5 days. On the fifth day they were transferred under the kidney capsule of ovariectomized hosts. Hosts were injected with 10 i.u. pregnant mares' serum gonadotrophin (PMSG Organon, Morden, Surrey, UK) 8 days post transplantation and 48 hours later injected with 10 i.u. human chorionic gonadotrophin (HCG) (Organon, Surrey). Animals were killed 12 hr after HCG injection and the grafts removed. Fine needles were used to disrupt the gels and the 8 largest follicles were dissected free. Oocytes were aspirated from the largest follicles and placed in a petri dish containing HEPES-buffered M199 medium. Sperm were obtained from a proven fertile F1 hybrid male and incubated at 37°C in Medium T6 (Quinn et al., 1982) for 3 hours prior to oocyte removal to fully capacitate the spermatozoa. Sperm were added to the dish containing the oocytes and sperm binding and penetration was observed using an inverted phase contrast microscope at x 400 magnification.

Results

Morphology of transplanted follicles:

After ovariectomy normal smear patterns were abolished and the vagina closed. The vagina began to open within 3 days of follicle transplant under the kidney capsule and was obvious by 5 days in 21 out of the 22 animals. The mouse killed 2 days after follicle transplantation did not exhibit vaginal opening and a vaginal smear taken by lavage at autopsy revealed few cells. Cornified epithelial cells were detected in the smears within 3 - 5 days and marked cornification of the vaginal epithelium was identified in the smears from day 7. Table 4.1. summarises the data from smears taken between 13 and 21 days after transplantation for 4 mice. These are representative of the pattern seen in the mice from about 9 days on: between 3-9 days the smears were more variable but cornified

cells tended to dominate. Normal oestrous cycles were not identified from the smears but all animals entered a period of persistent cornification. Vaginal atrophy persisted in the animals which received control transplants of collagen gel only: no vaginal opening was observed and so smears were not attempted. . Uterine weights showed a significant increase compared with control animals between 11 and 14 days after transplantation (Figure 4.1). The uterus from a gel-only control animal showed the atrophy expected after ovariectomy (Figure 4.2a.). The experimental uterine horn demonstrated the uterotrophic effects of oestrogen from the grafts (Fig 4.2b.).

Figure 4.3 shows follicles in collagen gel on the fifth day of culture, prior to transfer under the kidney capsule. As can be seen the gel contains a high density of follicles. The grafts were easily identified under the kidney capsule. All stages of follicle development were identified in the grafts from day 7 onwards (Fig 4.4). Thecal cells were observed in multilaminar follicles (Fig 4.5) and large antral follicles were present (Fig 4.6). No ovulations occurred from any of the grafts although follicles appeared to be mature and cumulus mucification occurred. The oocytes were mature, germinal vesicle breakdown occurred but the oocytes were not released and degenerated within the follicle (Fig 4.7). Luteal-like structures were also observed in the grafts (Fig. 4.8).

Observations on blood vessels and theca

After 3 days *in vivo* there was no evidence of either theca cells or blood vessels in the graft. By 5 days no theca was present but some small blood vessels were apparent. On day 6 both theca and blood vessels were present and by day 7 both were well represented.

Table 4.1. Record of the appearance of cells in vaginal smears from 4 mice taken between 13-21 days after transplantation of cultured follicles under the kidney capsule.

Mouse No.	Days after Transplant	Leucocytes	Nucleated Epithelial Cells	Cornified Epithelial Cells
1	13			+++
	14	+		+++
	15			+++
	16			+++
	17			+
	18	++		+
	19	+		+++
	20			+++
	21			+++
2	13			+++
	14			+++
	15			+++
	16			+++
	17			+++
	18			+
	19			+++
	20			+++
	21			+++
3	13	+	+	++
	14		+++	+
	15			+++
	16			+++
	17	no smear		
	18		+	+++
	19			+++
	20			+++
	21			+++
4	13	+++		+
	14	++		+
	15			++
	16	+		++
	17	no smear		
	18			+++
	19	++	+++	+
	20		++	++
	21	++		++

+ = few cells present, ++ moderate number of cells present, +++ cells very numerous.

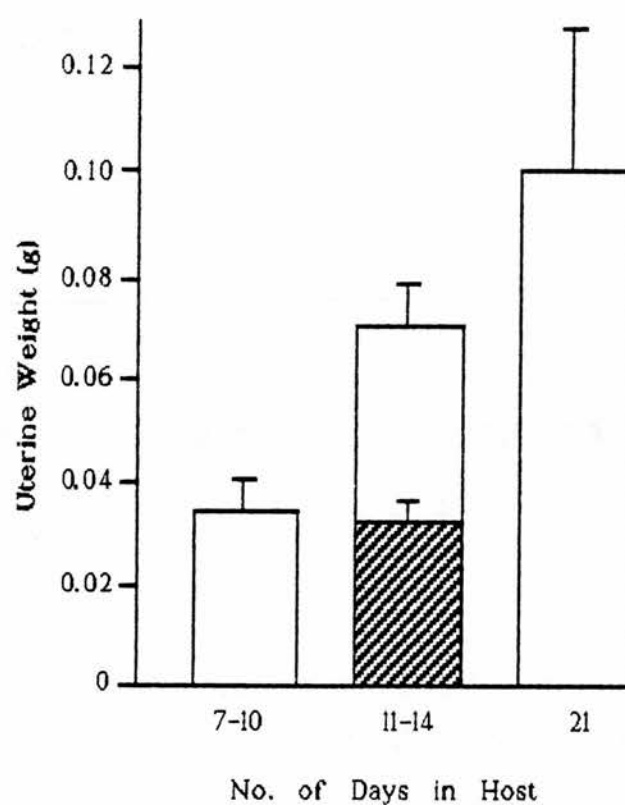


Figure 4.I. Histogram showing mean \pm s.e.m. uterine weight of ovariectomized hosts after transplantation of cultured follicles (open bar) or collagen gel only controls (hatched bar).

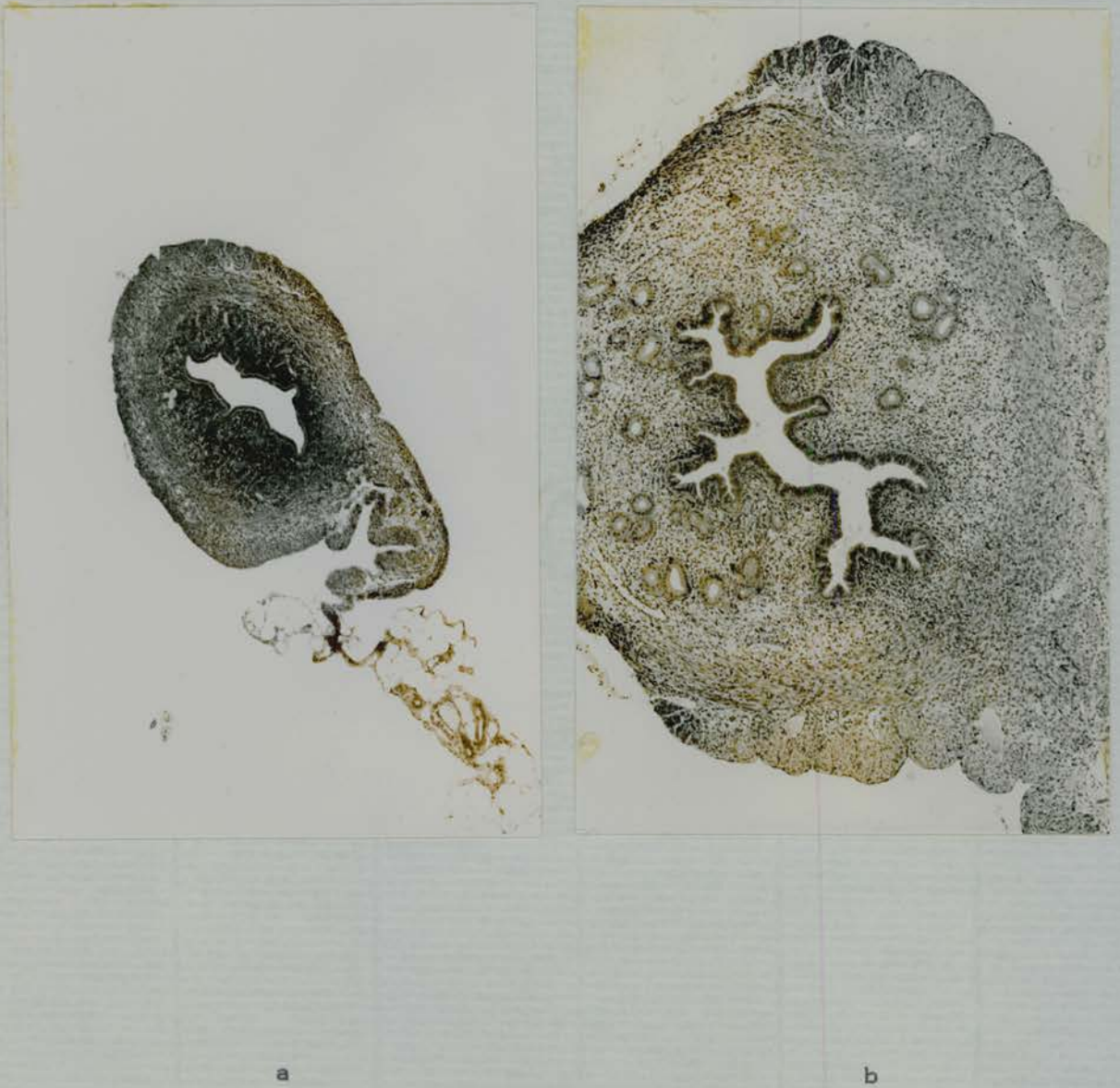


Figure 4.2. Photomicrograph of uterine cross-sections from ovariectomized mice (a) 21 days after gel-only (control) transplantation and (b) 21 days after transplantation of cultured follicles under the kidney capsule (x 50).



Figure 4.3. Follicles in collagen gel after 5 days in culture, immediately prior to transplantation under the kidney capsule. Follicle density is high (phase contrast x 100).

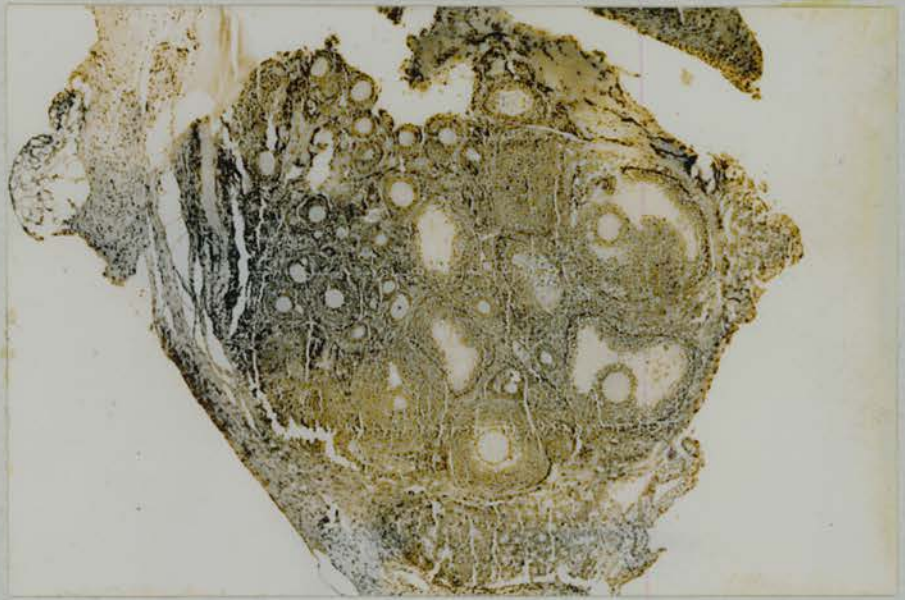


Figure 4.4. Section of a collagen gel grafted under the kidney capsule.

The follicles were cultured for 5 days before transfer and the graft removed after 13 days in the host. All stages of follicle development can be seen (x 50).

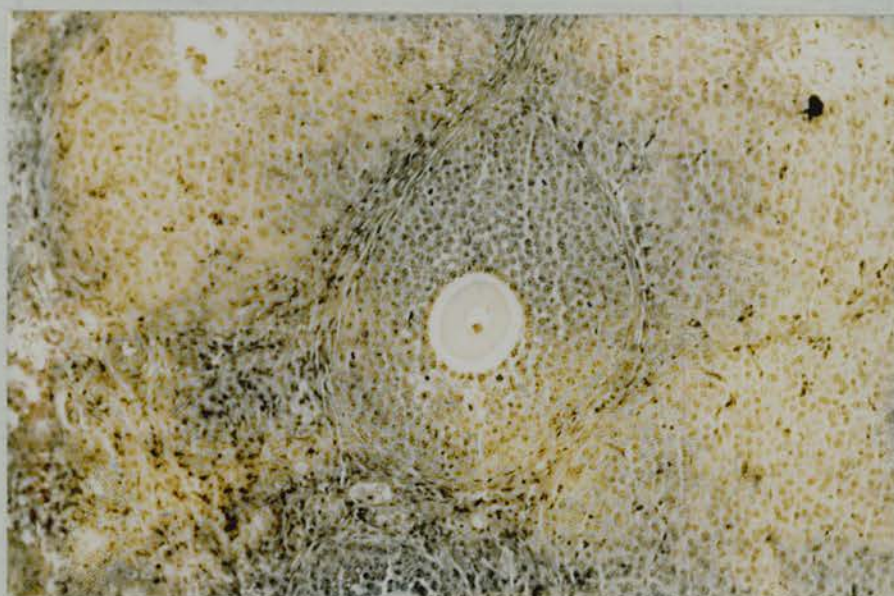
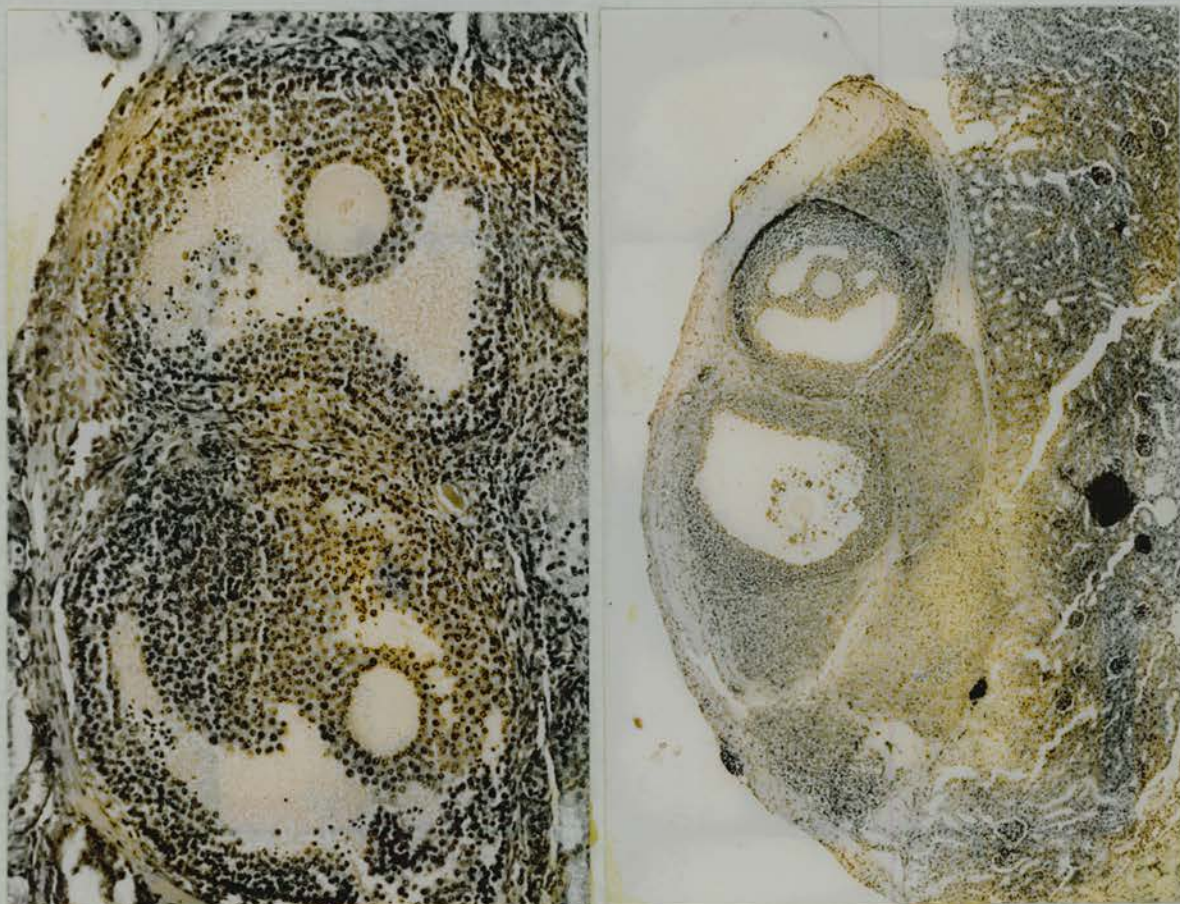


Figure 4.5. Section of graft showing a multilaminar follicle with defined theca layer. Follicles cultured for 5 days and graft removed after 13 under the kidney capsule (x 160).



a

b

Figure 4.6. (a) Section of graft showing large Graafian follicles after 5 days in culture and 13 days under the kidney capsule (x 160).

(b) Section of graft showing a large antral follicle and a degenerating Graafian follicle, 5 days in culture and 21 days under the kidney capsule (x 50).



Figure 4.7. Degenerating follicle with trapped oocyte. Follicles cultured for 5 days and transferred into host for 13 days (x 160).



Figure 4.8. Luteal like structures in a section of graft after 15 days in the host. Follicles cultured for 5 days before transfer (x 35).

Observations of oocyte sperm binding capability

All 8 oocytes observed showed sperm binding. Polyspermy was not observed. Culture conditions were not designed for *in vitro* fertilisation and oocytes were not observed after sperm binding had been demonstrated.

Discussion

In this study it has been shown that small preantral follicles, from immature animals, grown *in vitro* are capable of progressing to the mature Graafian stage when transferred *in vivo*. Within 5 days these transplanted follicles were producing sufficient oestrogen to support vaginal opening in ovariectomized animals and cornification of the vaginal epithelium was established within 7 days. These results would indicate that there is no inherent inability of collagenase isolated follicles to develop beyond the multilaminar stages and that the limiting factor to further development *in vitro* appears to be the culture conditions.

All stages of follicular development were present in the grafts. Even after 21 days *in vivo* primordial and small growing follicles of 1 and 2 granulosa cell layers were still present in the grafts. This suggests that these follicles had recently been recruited from the primordial pool. Collagenase dissociation and 5 days *in vitro* had not restricted the growth potential of the primordial follicles and possibly small growing preantral follicles. This was an encouraging result as it demonstrated that the isolation and culture procedures did not irreversibly alter the growth potential of the follicles, with development and improvement *in vitro* differentiation of theca and antral formation was a viable goal.

The grafts appeared to remain as discrete units under the kidney

capsule. There was no obvious growth of kidney cells into the graft and the grafts could be easily separated from the kidney tissue. Although not certain it seems likely that the cells which differentiated into theca were already present in the follicle culture. This suggests that the differentiation of the theca and the appearance of antra was due to the interaction of local factors in the graft with systemic factors of the *in vivo* environment and not to local interaction with the kidney tissues. Transplantation resulted in some injury and bleeding with a resulting inflammatory reaction. The inflammatory response could expose the graft to a number of growth factors and angiogenic factors (e.g. fibroblast growth factor, platelet derived growth factor, prostaglandins).

Theca differentiation is evident in 1-2 layered follicles in older mice but was not noted in the 10-day old ovaries used in this study and antrum formation occurs at days 13-15 (Gosden et al., 1988). The presence of a theca would appear to be necessary for further follicle growth. The steroidogenic capability of the follicle is compromised when theca layers are absent (Richards & Kersey, 1979). It has been suggested that theca cell differentiation is related to subtle increases in serum LH levels (Bogovich & Richards, 1982). Stromal cells, the presumed precursors of theca, were present in the culture system but did not differentiate into thecal cells *in vitro*. The results of our study show theca developing only after the appearance of blood vessels in the graft. A blood born factor or perhaps the production of a growth factor such as FGF by the endothelial cells might be involved. Qvist et al. (1990) placed preantral follicles with a rudimentary theca into culture and reported incomplete thecal formation *in vitro*. The *in vitro* conditions which included supplementation with 50% serum supported antrum formation but were not able to maintain normal thecal development. The serum used in the culture medium in the present

study was not characterised for growth factors but might be expected to provide a range of factors. The *in vitro* conditions were obviously inadequate for initiating/supporting thecal induction.

Interactions between endothelial and stroma cells could be a factor in theca differentiation, a hypothesis that may be tested by co-culturing follicles and endothelial cells. Alternately it may simply be blood borne factors, not present in donor calf serum, that are critical. The possibility of endothelial cell-follicle interactions merits further study.

The ovarian follicle develops an extensive network of blood and lymphatic vessels in the theca interna as folliculogenesis progresses. As one of the few sites in the body where normal, non-traumatic angiogenesis occurs, the ovary has an important role in investigation of the physiological rather than pathophysiological regulation of neovascularisation and a number of putative angiogenic factors have been identified (Koos, 1989). The theca can stimulate endothelial cell proliferation (Makris et al., 1984). Granulosa cell-conditioned medium also stimulated endothelial cell proliferation and the formation of capillary-like tubules *in vitro* (Koos, 1986; Blake & Koos, 1987). The angiogenic actions of ovarian tissue extract are enhanced by gonadotrophin administration (Sato et al., 1982). A range of possible angiogenic factors are produced by the granulosa cells: oestrogens, basic fibroblast growth factor (bFGF), transforming growth factor- β and others (see Koos, 1989). A more detailed discussion of the role of growth factors is presented in chapter 6. FGF, which is also produced by endothelial cells (Schweigerer et al., 1987), may also have a role in regulating granulosa cell cytodifferentiation, possibly via interactions with ECM components such as GAGs (Neufeld et al., 1987; Gospodarowicz, 1989; Koos, 1989). It may be that a complex interaction between stroma, endothelial cells and granulosa, possibly mediated by

growth factors, is required for full induction of the theca.

Ovulation did not occur from the grafts: oocytes degenerated within the follicle and granulosa cells luteinised. This pattern of follicular degeneration has been observed in the rat ovary with LH-RH agonists (Popkin et al., 1983) and in the mouse after suppression of ovulation by progesterone (Telfer et al., 1990a). Felicio et al. (1983) have reported ovulation from intact ovarian fragments transplanted under the kidney capsule and it is possible that the lack of an organised ovarian surface epithelium in the grafts would explain the differences. The role of the surface epithelium has been discussed in chapter 1.

Sperm binding was demonstrated by oocytes taken from follicles grown *in vitro* and then transplanted under the kidney capsule. Later work has demonstrated that blastocysts can be produced from oocytes using this system (Telfer et al 1990b). Follicle-gels were transferred to PMSG and hCG primed hosts and the grafts harvested 8 days later. Fine needles were used to free the follicles and the oocytes were aspirated from the largest follicles and fertilised *in vitro* by established techniques. 16 oocytes were recovered and fertilized, 12 developed to the 2-cell stage and four formed blastocysts. That embryos were formed shows that the culture of immature follicles does not irreversibly impair their developmental potential. Using the same culture and transplantation method with frozen preantral follicles Carroll et al. (1990) have produced live young. Eppig & Schroeder (1989) have used *in vitro* fertilization to produce live young from oocytes grown and matured in culture.

The techniques of follicle isolation and culture described in the last chapter are potentially powerful tools for investigating many aspects of follicle growth. Combining the collagen gel system with *in vivo* assessment after transplantation provides an essential assessment of follicle potential.

Used together the techniques may overcome some of the limitations of the *in vitro* system.

CHAPTER 5

An autoradiographic investigation of hormonal effects on in vitro
development of mouse ovarian follicles.

Introduction

The purpose of the work reported in this chapter was to utilise the culture system to investigate the influence of paracrine or autocrine factors on preantral follicle development. The traditional concept of endocrinology concentrated on the production of a chemical messenger or hormone in a group of endocrine cells, the release of the hormone from the cells and its transport via the bloodstream to a distant target tissue. The rapid development of the cell culture approach and in particular the investigation of endocrine cell differentiated function *in vitro* has led to the realisation that hormones may have local endocrine functions. Paracrine messengers act by diffusing locally to the target cells (Dockray, 1979). Autocrine regulation involves the production and secretion of a chemical messenger by a cell, with the released messenger then acting via receptor binding to regulate cellular function. This concept of the secreting cell also being the target cell was derived from cell culture studies of transformed cells (Sporn and Torado, 1980). Intrafollicular factors using autocrine and paracrine mechanisms are thought to be important in regulating ovarian function but as with other aspects of follicular physiology much of the available evidence related only to the antral follicle or to studies using granulosa cells, especially from the oestrogen-treated immature or hypophysectomized rat, in monolayer cultures (reviewed by Hsueh et al., 1983; Hillier, 1985; 1987; Westergaard, 1988). Three main groups of possible paracrine factors were identified from the literature: the classically-defined endocrine hormones, for example oestrogens, progesterone and androgens, other locally produced factors such as the prostaglandins and the more recently identified peptide growth factors such as epidermal growth factor, transforming growth factor β and

fibroblast growth factor (Barnes et al., 1987).

Early studies of endocrine cells in culture revealed difficulties in demonstrating unequivocal effects when hormones were added to the culture medium and the use of serum in the culture medium was identified as a potential problem (Hayashi and Sato, 1976). Serum contains a range of hormones and growth factors which may modulate cell growth and/or differentiation and the effects of exogenously added hormones or growth factors can be masked by the presence of serum. Serum can have an inhibitory effect on granulosa cell cytodifferentiation, with formation of steroidogenic enzymes and LH receptor induction being blocked (see Erickson, 1983). A strategy was adopted which used two approaches.

To test the role of selected steroid hormones and to demonstrate that the culture system was able to support experimental modulation of follicle development, the role of oestrogen and follicle stimulating hormone was investigated using serum-supplemented medium. This necessitated the development of techniques using autoradiography and image analysis to assess follicle function. At the same time the value of a serum-free culture system was recognised and alternatives to serum supplementation were investigated.

In this chapter the role of the classical hormones in small follicle development and the use of autoradiography and image analysis in analysing these effects is presented. The development of a serum-free medium and the utilisation of this medium in the investigation of peptide growth factors is reported in the next chapter.

The potential role of hormones in preantral follicle function has been introduced in chapter 1 (page 22). Studies of hypophysectomized and homozygous *hypogonadal* (*hpg/hpg*) mice indicate that the gonadotrophins are not obligatory for small preantral follicle development (Faddy et al.,

1976; 1983; 1987; Halpin et al., 1986) but it appears that the gonadotrophins may modulate preantral growth (rat - Arendsen de Wolff-Exalto, 1982; hamster - Roy and Greenwald, 1986; 1989). Arendsen de Wolff-Exalto (1982) transplanted neonatal rat ovaries, obtained on the day of birth into adult female hosts. The hosts were either ovariectomized to produce high levels of gonadotrophins or ovariectomized and hypophysectomized resulting in very low gonadotrophin levels. The grafts were harvested after 15 days. The study did not indicate any significant difference between the two groups for the total number of follicles but grafts exposed to a high gonadotrophin environment had more, larger preantral follicles. In follicles containing 6-7 granulosa cells there was a small but significant increase in the number of cuboidal granulosa cells in the gonadotrophin exposed grafts. This difference disappeared in follicles with 8-9 granulosa cells and at the 10 cell stage all the cells were cuboidal. The hormone-rich grafts also showed a higher percentage of oocytes with a diameter $> 21 \mu\text{m}$ and follicles with less than 7 cells contained significantly larger oocytes than in the gonadotrophin-deprived grafts. The larger follicles also had a better developed theca than the low gonadotrophin level group.

The granulosa cells of the preantral follicle have receptors for FSH and de Reviers (1988) has demonstrated that exogenous FSH increases granulosa cell thymidine incorporation in gonadotrophin-deficient dwarf mice. Granulosa cells from large preantral follicles obtained from oestrogen treated, hypophysectomized immature rats also demonstrated FSH-responsiveness (Hsueh et al., 1983). Roy & Greenwald (1989) reported that preantral hamster follicles *in vitro* responded to FSH with an increased labelling index and reduced atresia. From these studies it appears that FSH can exhibit an influence on preantral development, either by acting

directly or by interactions with other hormones. The local effects of the ovarian steroid hormones have been extensively studied in larger follicles both *in vivo* and by using cultured granulosa cells. The effects of the ovarian steroids on preantral growth has received less attention and their role in the preantral follicle remains uncertain.

Oestrogen has been shown to stimulate granulosa proliferation, increase follicular size, increase the number of gap junctions between the granulosa cells and reduce atresia (Payne and Hellbaum, 1955; Goldenberg et al., 1972; Merk et al., 1972; Harman et al., 1975; Burghart & Anderson, 1978; Hillier and Ross, 1979, Hsueh et al., 1983). *In vitro* studies of granulosa cells obtained from this oestrogen-stimulated hypophysectomized rat model indicate interactions between oestrogens and FSH (Hsueh et al., 1983; Erickson et al., 1983). Species differences are probably important. Oestradiol did not influence *in vivo* follicular development in the hamster (Kim et al., 1984) or the monkey (Keoring et al., 1986). In a long-term culture system, Roy & Greenwald (1989) reported that oestradiol had no influence on the labelling index and induced follicular atresia in hamster follicles with 1-3 granulosa layers. Oestrogen implants have been reported to decrease follicle atresia in the immature rat (Gondos, 1982). More recently exogenous oestrogen has been shown to increase the numbers of medium to large sized preantral follicles in the mouse (Kim & Greenwald, 1987)

Studies of androgens suggest that they inhibit preantral follicular development and induce atresia (Payne et al., 1956; Payne & Runser, 1958; Louvet et al., 1975a Zeleznik et al., 1979; Hillier and Ross, 1979). Concurrent administration of oestrogen has been reported to oppose androgen induced atresia (Gondos, 1982). Mitchell et al., (1975) also report an anti-atretic action of oestrogen in the rat. The effect of progesterone

on small follicle development is also uncertain. In the hypophysectomized rat and hamster large doses of exogenous progesterone can inhibit oestrogen-stimulated follicular growth (Payne et al., 1956; Chiras & Greenwald, 1978). Pedersen & Peters (1971) reported reduced recruitment but no effect on follicular growth rate while Telfer et al. (1990a) using progesterone implants reported an increase in the rate of preantral follicle growth. Roy & Greenwald (1989) reported a slight enhancement of the labelling index in small preantral follicles of the hamster by low levels of progesterone but the effect was not sustained.

The use of radiolabelled tracers to investigate a number of aspects of follicular function is well established. A number of studies have used labelled thymidine to investigate mitotic activity in the granulosa cells (Pedersen, 1970a,b; Hoage & Cameron, 1976; Hirshfield, 1989), labelled hormones have been used to identify and localise hormone receptors (Nimrod et al., 1976; Oxberry & Greenwald, 1982; Mulheron et al., 1987), radiolabelled nucleosides have been used to determine oocyte-granulosa cooperation (Heller & Schultz, 1980; Moor et al., 1980; Motlik et al., 1986) and labelled amino acids to assess metabolic activity and the influence of hormones (Mori et al., 1982; George & Wilson, 1978; Tjugum et al., 1984). Autoradiography, the use of photographic emulsions to detect the ionizing radiation released by the radiolabelled tissues, provides a particularly useful approach in that it allows the distribution of the radiolabelled substance to be identified. Autoradiography can be used to investigate distribution of the labelled compound between the three follicular compartments - oocyte, granulosa and theca. [^3H]thymidine incorporation by granulosa cells as a marker for cell proliferation is probably the most widely used application of autoradiography in follicular studies. The present study used autoradiographic assessment of [^3H]methionine

incorporation as an indicator of follicular protein synthesis. Methionine as one of the essential amino acids cannot be synthesised by the follicular cells but is a component of most cellular proteins. The ^3H isotope is useful as it is a weak beta particle emitter, the short path-length of the beta emissions in the photographic emulsion is useful for localisation of activity.

Autoradiography is useful for mapping the distribution of a tracer within a tissue using light microscopy to identify the silver grains. Quantitative autoradiography tries to measure cellular activity, but to achieve this it is necessary to be able to define the relationship between the number of silver grains per unit area and the amount of ionizing energy present in the tissue (the number of radiolabelled molecules present) with some precision. Optical densitometry is one approach but with the development of computer assisted image analysis systems a more flexible approach is possible. Computer-assisted image analysis, qualitative and quantitative autoradiography have been widely applied in the fields of neuroanatomy and neurophysiology (e.g. 1984; Benfenati et al., 1986; McCasland & Woolsey, 1988) but application of the image analysis approach has been limited in the study of reproductive physiology. Image analysis has been used to quantify oestrogen receptor content and monoclonal antibody staining in breast carcinoma (Franklin et al., 1987; Parham et al., 1989; Horsfall et al., 1989). Schipper et al., (1989) reported an interesting application of the technique in the estimation of the percentage of epithelium and stroma in ovarian tumour tissue.

The approach used in this study, localisation and semi-quantitative assessment of the incorporation of [^3H]methionine by liquid emulsion autoradiography and video image analysis, was adopted because it allowed the effects of hormonal treatments to be investigated by measurement of

follicle and oocyte area, localisation of the label in oocyte and granulosa compartments and a semi-quantitative estimation of the density of silver grains. This application of autoradiography and image analysis is a novel approach in the field of follicular physiology.

MATERIALS AND METHODS

Animals

The mice used in the following experiments were F₁ females aged 8-11 days post partum and derived from matings between CBA/Ca males and C57Bl/6 females. Breeding pairs were provided with a pelleted diet and water *ad libitum* and housed under light for 14 hours starting at 0700 GMT. Room temperature was maintained at 21°C. The immature mice were used immediately after their removal from the mother. Twenty-four animals were used for each experiment.

Pilot Experiments

Before the main study was commenced some pilot work was carried out to establish a suitable protocol and gain familiarity with the techniques. The literature was searched to suggest an appropriate range for the molecular concentration and specific activity of [³H]methionine for the *in vitro* labelling. A 3 hour incubation period and one weeks exposure after coating with photographic emulsion were desirable objectives. Follicles were isolated and cultured as described in chapter 3. Radioincubation was carried out 3 hours before the gels were harvested for histological processing. Three levels of tritiated methionine were tested using methionine-free medium - 0.2, 1 and 5 µCi/ml (Amersham International, specific activity 85Ci/mmol). At this point the image analysis system was not available and the number of silver grains in the autoradiographs was

assessed by simple light microscopy. The highest level of radiolabelled methionine, 5 $\mu\text{Ci/ml}$, provided a distinct number of silver grains in the granulosa and oocyte. If this was taken as the baseline then the number of grains present offered scope for detecting both increases or decreases in the grain density after hormonal treatment. A higher density of grains might have made it difficult to measure any increase in grain density after hormonal stimulation as the image analysis system would be unable to identify discrete grains.

The effect of staining on the image analysis procedure was also investigated. The following were assessed: 1) unstained, 2) eosin, 3) methyl green-pyronin and 4) a light haematoxylin and eosin stain. The first problem with the unstained tissue was that of locating and moving between sections. Even with phase contrast it was time consuming to locate the sections. It was difficult to judge the histological quality of the follicles and to visualise and draw round the follicle with the mouse was also a problem for the same reason. Haematoxylin & eosin and methyl green-pyronin staining made the follicles easier but the background colour of these stains and the darker staining of nuclear material made it difficult to produce a sharp contrast between the background and the silver grains. The use of a light eosin stain and of a red filter on the microscope provided a compromise solution. The tissue was easily located, the oocyte nucleolus was evident and measurements of total areas and grain areas could be carried out. The main limitation was that assessment of follicle quality was still difficult, granularity of the cytoplasm, separation of granulosa layers and contraction of chromatin were not well distinguished by the light eosin stain.

The effect of oestradiol and FSH on follicular growth

Culture Media

All preparatory steps were carried out in HEPES-buffered medium M199 (Gibco) and follicles were cultured in bicarbonate-buffered M199 with Earles salts (Gibco) containing 10% donor calf serum (Flow Laboratories). The serum had been heat-inactivated for 35 minutes at 55°C. Both media were supplemented with gentamicin 100 µg/ml (Sigma), amphotericin B 2.5 µg/ml (Sigma) L- glutamine 100 µg/ml (Flow Laboratories) and sodium pyruvate 35 µg/ml (BDH).

Follicle isolation and culture

Follicle isolation and collagen gel culture were carried out as described in chapter 3. All steps were carried out using ice-cold preparation medium. The bisected ovaries were incubated with gentle rotation (1 Hz) at 37°C in a 10ml tube containing Hepes-buffered medium with 1.5 mg/ml of collagenase (Sigma type I) and 40 units/ml of DNase I (Sigma) for 30 minutes. After removal of the collagenase by gentle centrifugation the follicles were freed by repeated pipetting. The isolated follicles were pelleted by gentle centrifugation, the supernatant removed and the follicle pellet resuspended in 3 ml of medium and concentrated in the centre of the watchglass by gentle rotation. To increase the number of follicles per gel, 30 µl samples were pipetted from the central concentration into the wells of a Terasaki plate (Flow Laboratories) and the rotation and pipetting were repeated until 12 samples had been obtained from the area of concentrated follicles. The follicles were set in collagen gel derived from rat tails as before.

The twelve gels were transferred to the wells of a Linbro tissue

culture plate (Flow Laboratories). Four wells containing 2 ml medium were pre-incubated to warm and gas the medium and the gels randomly assigned, 3 to a well. The gel cultures were incubated at 37°C with 5% CO₂ in a humidified incubator. The medium was changed at 48 hours and on the fourth day the medium was removed and replaced by hormone-supplemented culture medium as outlined below. Three hours before the termination of the cultures on day 6 the medium was removed and the gels rinsed twice with methionine-free medium 199 (Gibco). Then 0.25ml per gel of ¼-strength methionine medium supplemented with serum, the appropriate hormones and containing 5 µCi/ml ³H-Methionine (Amersham International, specific activity 85Ci/mmol) was added. The follicles were returned to the incubator and cultured for three hours. At the end of the culture period the radioactive medium was removed and the gels rinsed twice with normal culture medium. The gels were then fixed in Bouin's fluid and processed for autoradiography.

Hormone supplementation

On the fourth day the culture medium was replaced with hormone supplemented medium. Each well was randomly assigned a different treatment. One well received the usual culture medium and served as a control. The three experimental treatments were oestrogen (1 µg/ml, Sigma), ovine FSH (20 ng/ml, NIADDK-ofsh-16, NIH, Bethesda, USA), or oestradiol 17β (1 µg/ml) and FSH (20 ng/ml). A duplicate experiment was performed. The steroid was prepared in ethyl alcohol and then diluted to give a final concentration of less than 0.01% alcohol in the culture medium.

Autoradiography

After 24 hours in Bouin's fluid the gels were dehydrated and embedded

in paraffin wax (Appendix 1). Serial sections were cut at 5 μ m and mounted on acid-cleaned, gelatin-subbed slides. "Chance" glass slides which have produced results with low background counts were used (R.G Gosden, personal communication). Fresh alcohols and wax were used at each step. The sections were dewaxed in fresh xylene and brought down to water through an alcohol gradient. They were then washed thoroughly in tap water for thirty minutes and then in two changes of freshly distilled water. Slides were placed back-to-back and in the darkroom coated with Kodak K2 Nuclear Emulsion diluted 50% with distilled water. One operator carried out the dipping to minimise variation. The room temperature was monitored as variations in room temperature can affect background activity. The slides were allowed to dry for one hour in a light-tight box. They were then transferred to light-tight plastic slide boxes containing silica gel desiccant, and sealed with black tape. After exposure at 40°C for one week the slides were developed in Kodak D19 developer and fixed with Ilford Hypam Fixer. To minimise variation due to autoradiographic processing, duplicate experiments were dipped and processed for autoradiography together. Sections were lightly stained with eosin and mounted with DPX. Control slides from a non-labelled tissue and from a labelled tissue were included in each batch.

Image Analysis

Image analysis was carried out using a dedicated microcomputer-based image processing and analysis system, Image Manager, supplied by Sight Systems, Newbury, UK. The system consists of an 20Mb XT IBM-compatible microcomputer (Epson AX2 PC), two Philips colour monitors (one for displaying program information, the other for the video image), a Hitachi KP143 medium resolution solid state CCD video camera, control mouse,

image manager unit PC 1, Video contrast enhancer CE 1 and Freelance (a general-purpose image analysis program). Figure 5.1. is a diagram summarising the main hardware and connections. The video camera was mounted on a Leitz Diaplan microscope. Image analysis was carried out using the x40 objective lens. The PC1 image manager unit, CE 1 contrast enhancer and the Freelance program were manufactured by Sight Systems and performed the main image handling functions.

The image manager contains two independent framestores. The first framestore is a grey store which can be of up to 7 bits resolution giving a maximum of 128 grey levels and a 512 by 512 pixel grey image. The second framestore permits a binary image (a 512 by 512 pixel binary image) to be extracted without loss of the original grey image. The binary image can be frozen and used for subsequent manipulation by the computer using the Freelance image analysis program. The video output utilises an 8 bit digital mixer which allows the grey image and binary image to be viewed separately or with the binary image overlaid. The contrast enhancer unit CE 1 plugs into the PC 1 unit and allows the operator to improve the contrast between darker features (the silver grains in this case) and the general background (eosin-stained cytoplasm).

Freelance is a menu-driven image analysis program responsive to both keyboard and mouse commands. The program can be used in an interactive mode or to generate a task file containing a sequence of operations with prompts for the operator which can be stored and run as required. Freelance offers a number of options or tools for image analysis. The tools utilised in the task files created for follicle measurements were control, environment, image capture, thresholds, binary image operators and measurements. The control menu does not operate in the interactive mode but is used when creating a task file. It is used to repeat sections

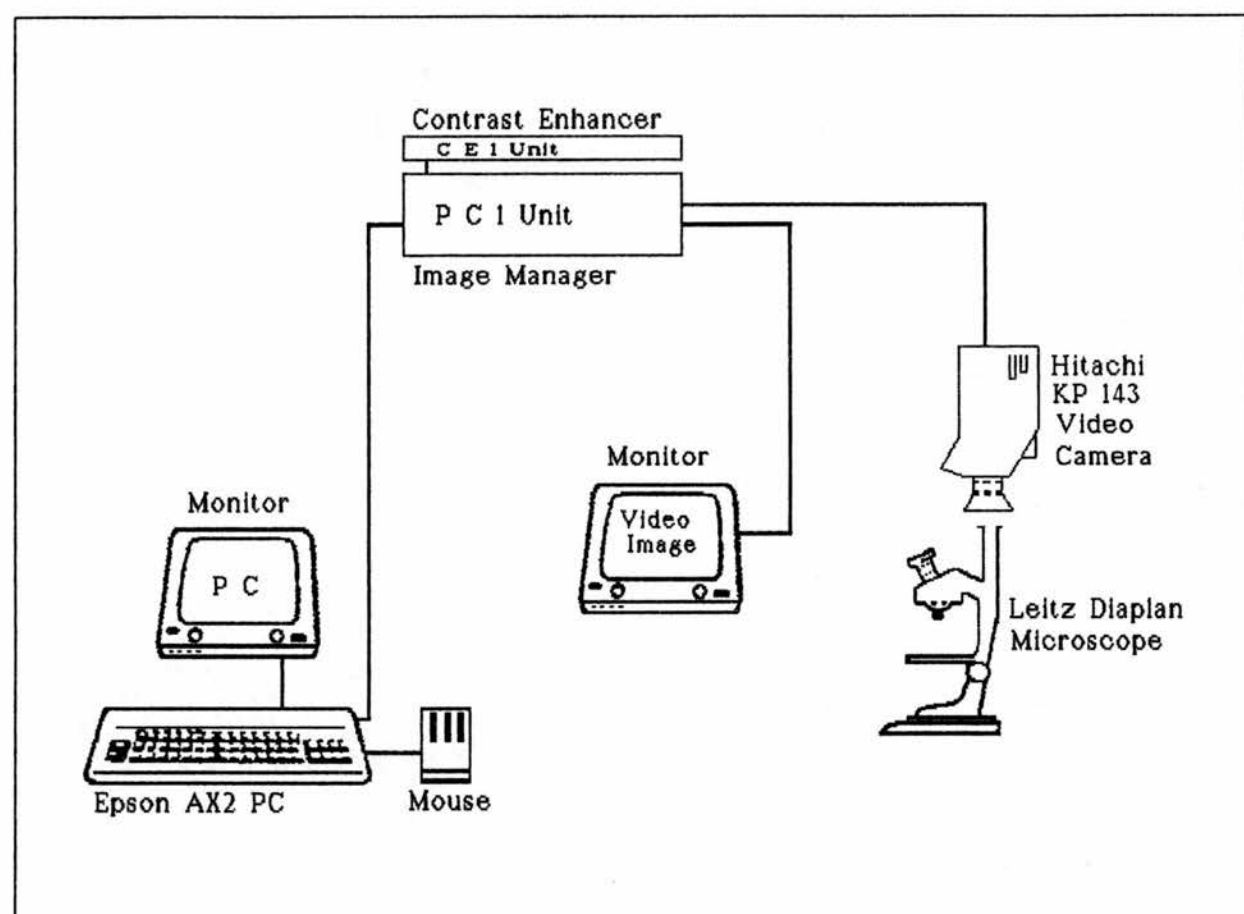


Figure 5.1. A diagrmtic representation of the main hardware components of the image analysis system. A 20Mb hard disk and printer were also included and a Mitsubishi Video Copy Processor Model P60B was attached when instant, hard copy of the screen image was required.

of the task file to allow multifield and multisample analysis to be performed. The environment menu offers a range of options which enable the user to set up the conditions for analysis - to calibrate the system and define the region(s) for measurement. The calibration option allows units of measurement to be defined and the specification of x and y calibration. Default calibration utilises a captured image but previously defined scaling factors can be entered manually. The size and shape of the measurement frame can be altered or the analysis region defined. The analysis region can be the whole image, the defined frame or an arbitrary region drawn using the mouse. Freehand line or segments can also be designated the analysis region.

The image capture menu facilitates capture of a live or stored image and thresholding of the whole image. The live option displays the live image from the video camera and brightness allows the brightness of the image to be adjusted to optimise the range of grey levels. Thresholding of the live image can be performed. The thresholds menu creates a binary image from the captured grey image and in this menu thresholding is performed over the region for analysis only. Thresholding allows the desired features to be highlighted by overlaying them with red pixels. Binary image operators allow the binary image to be edited with functions such as draw, erase, reject and others. The measurement menu is used to create a data file and select parameters for measurement. Two types of measurements are made, field and object measurements. Field measurements include area (the area of the measurement frame) and detected area (the total area overlaid by red pixels). These are relatively fast measurements and are expressed in the calibrated units.

The range of object measurements is wider and includes area and perimeter, detected area, width and height, length and breath, orientation

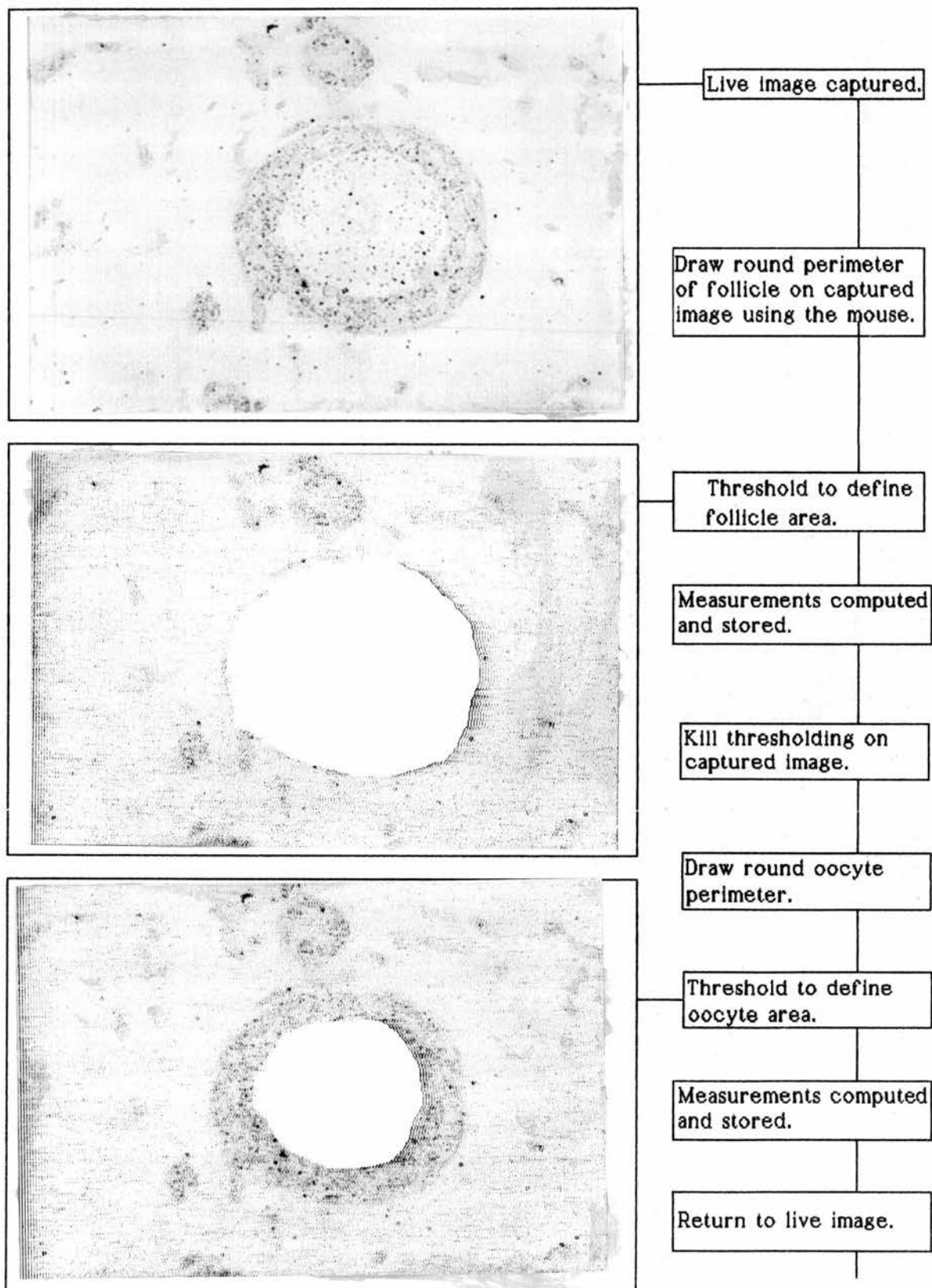
and integrated (optical) density. Area and perimeter measurement of the individual highlighted objects is a fairly fast measurement. Detected area is the number of red pixels making up the object. Object width and height are the projections of the object onto the x and y axes and are fast measurements. Object length and breadth give the maximum object length and the breadth; the breadth is the maximum breadth as projected onto an axis orthogonal to that of the length: these are relatively slow measurements. Orientation gives the orientation of the length axis in radians relative to the bottom of the image. Integrated density is an uncalibrated measurement of the optical density of each pixel. The measurement menu allows any of the options to be selected or rejected.

Two task programs were created to measure incorporation of tritiated-methionine as assessed by autoradiography. The first file was designed to measure follicle and oocyte area and the area over the granulosa compartment and oocyte occupied by silver grains. The second file was created to assess background autoradiographic activity by measuring the area occupied by silver grains in a region of the collagen gel which was free of tissue.

Assessment of the follicles

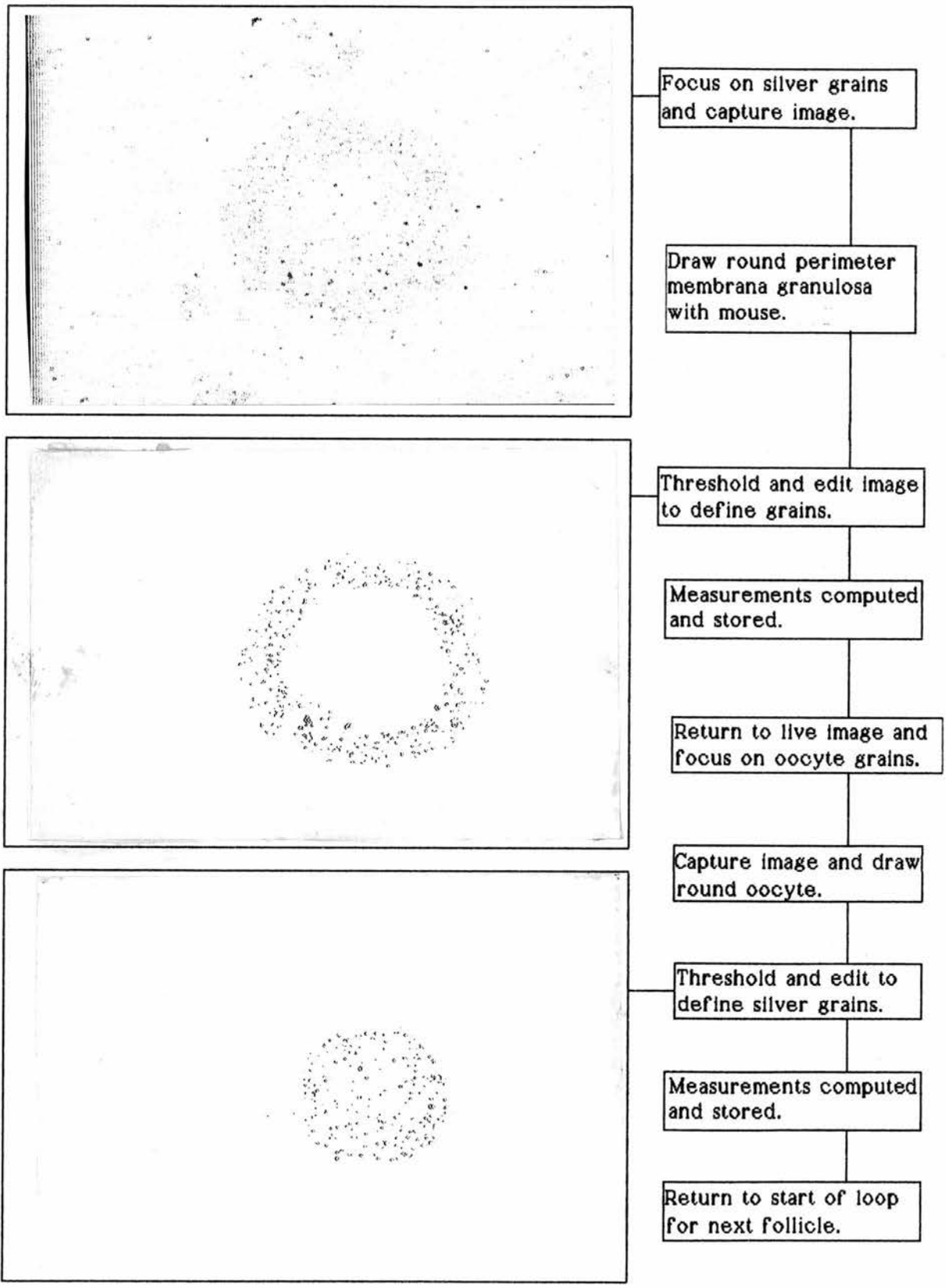
The task file for follicle measurements is summarised by the flow diagram Figure 5.2. The first step is to use the control function to set up a loop allowing 20 follicles to be measured by the task file. The environment mode was then used to calibrate the system. A 1 mm x 100 divisions graticule (Graticules Ltd, Tonbridge, Kent, UK) was viewed with a x40 objective lens to calibrate the x-axis. The graticule was then viewed at right angles to the x-axis and y axis calibration performed. After calibration a live image of the follicle is captured and in the environment

Figure 5.2. Major steps in the task file for measuring follicle and oocyte areas and [^3H] methionine incorporation as assessed by the % of membrana granulosa or oocyte area occupied by silver grains. Images obtained using a Mitsubishi Video Copy Processor.



Continued on next page.

Figure 5.2. - Continued.



mode a the region for analysis is defined using the mouse to draw round the perimeter of the follicle. Thresholding is automatically set to maximum upper range which overlays the whole of the follicle with red pixels, treating it as a single object. Measurements are then carried out of object detected area, object detected width and height. Thresholding is then removed and the user returned to the environment menu and prompted to draw round the oocyte perimeter. The whole oocyte is then marked by thresholding and measurements of detected area, width and height recorded.

The task file then returns to a live image and the microscope can be focused to maximise the silver grain image. Contrast can be enhanced using the CE1 unit, if required. The image was captured and the granulosa cells defined as the region for analysis, using the mouse. The thresholding option was then invoked and thresholding adjusted until the silver grains were overlaid by red pixels. The field detected area measurement was carried out to calculate the area covered by red pixels. Object measurements were also obtained of grain(s) area, width and height. The number of objects (grains or groups of grains) was also recorded. The image was then returned to live and the microscope focused on the grains over the oocyte. The oocyte is outlined as the region for analysis and thresholding and measurements repeated. The loop repeats until 20 follicles have been measured.

Measurements were made on follicles Stage II to Stage IV. Stage II follicles were further classed as Stage IIa (small follicles with a single layer of granulosa cells) and Stage IIb (large follicles with a single layer of granulosa cells). Measurements were carried out on up to 20 follicles of each stage. A follicle was only measured when the oocyte nucleolus could be distinguished i.e in an approximately equatorial section of the follicle. Four measurements were subtracted from the data files for

analysis - follicle area, oocyte area, total area of silver grains over the granulosa cell compartment, total area occupied by silver grains over the oocyte. Granulosa cell area was calculated by subtracting oocyte area from follicle area and the grain area expressed as a percentage of the granulosa or oocyte area. Image analysis was carried out blind. The different treatments were coded by the histology technicians during processing and only decoded after the measurements had been carried out.

The reliability of the measurements was assessed by recounting a single stage IV, III and IIb follicle 10 times each. The counts were carried out over a period of a week with at least 30 minutes between each count.

Assessment of background activity

All the gels from the two replicate experiments were coated with autoradiographic emulsion together to minimise variation between them and allow the gels to serve as controls for autoradiographic background activity. Sections of collagen gel which did not contain any tissue were used to assess background activity and ensure that any variation between the experimental treatments was not due to variations in the background. A task file was created to measure the background grains in such areas. A loop of 20 measurements was created and the system calibrated using the scaling factors obtained from the follicle task file. The environment menu was used to create a circular frame of 7736 μm^2 area and this frame was defined as the region for analysis. The tissue section was located and then viewed under the x40 objective. An area of gel free from tissue was located and the microscope focused to identify any silver grains. The image was captured and the pre-defined frame superimposed. The grains were highlighted by thresholding and measurements of object number, object area and total area of red pixels (field detected area) were carried

out. The frame was then moved to another section of the image and the process repeated. Background was assessed on 10 tissue sections (2 measurements per section) from each treatment group. From the data files the area of grains and number of grains were subtracted for analysis. As the same frame area was used for each measurement the detected area measurement was used without conversion to a percentage of the frame area.

Analysis

Data from the replicate experiments were pooled and analysed using the SPSS/PC+ statistical analysis program (Chicago, Illinois, USA). Descriptive statistics and two-way analysis of variance were calculated for the follicle measurements. One-way analysis of variance was used to assess the data from measurement of background activity. Multiple comparisons were carried out using the Tukey test with unequal sample size as described by Zar (1984). The $p = 0.05$ level of significance was chosen.

Results

Daily assessment of the follicle culture by inverted phase microscopy revealed no difference between the treatments and follicle growth appeared to be progressing normally. The processing of all the slides from the two replicate experiments together reduced one source of autoradiographic variability but one problem with the autoradiography was noted. When sections or ribbons were very close to the edge of the glass a higher density of grains was noted. This was found in all treatment groups and was judged to be an artefactual problem, possibly due to excess emulsion collecting at the edges during drying. The problem was overcome by only counting sections away from the margins of the slide.

Figure 5.3. summarises the data for the follicle Stage IIb - Stage IV follicle and oocyte area reproducibility counts. Figure 5.4. shows repeated measurements of the percentage area covered by silver grains for follicle and oocyte. Ten measurements were made of each follicle. Measurements of follicle and oocyte areas were found to be very reproducible, with coefficients of variation ranging from 3.28 - 4.63%. Coefficients of variance for grain areas ranged from 12.70 - 29.19%.

Table 5.1 lists the means for 40 measurements of background activity in tissue-free areas of collagen gels from each of the experimental groups. One-way analysis of variance indicated that there was no significant difference between the background activity for the four treatment groups. The sections chosen for measurement were also used for the follicle measurement and, as the treatments were all processed for autoradiography in a single batch, grain area of the tissue-free collagen gel should provide a good assessment of non-specific binding of the radiolabel and other sources of background radiation.

Two- way analysis of variance indicated a significant effect of both follicle stage and hormone treatment on follicle, membrana granulosa and oocyte area. A significant interaction between follicle stage and treatment also occurred for follicle and oocyte area but not for membrana granulosa area. Table 5.2. summarises the mean follicle area for each of the 4 treatments and follicle stages. Multiple comparison testing using Tukey's HSD (Honestly Significant Difference) test produced the expected significant differences at the $p = 0.05$ level between follicle stages and follicle area. Regardless of hormone treatment follicle area measurements followed the pattern: Stage IV > III > IIb > IIa. Area measurements of Stage IIa follicles show no stimulatory effect of FSH but indicated an inhibitory effect from oestradiol 17 β (E₂): control was not significantly different

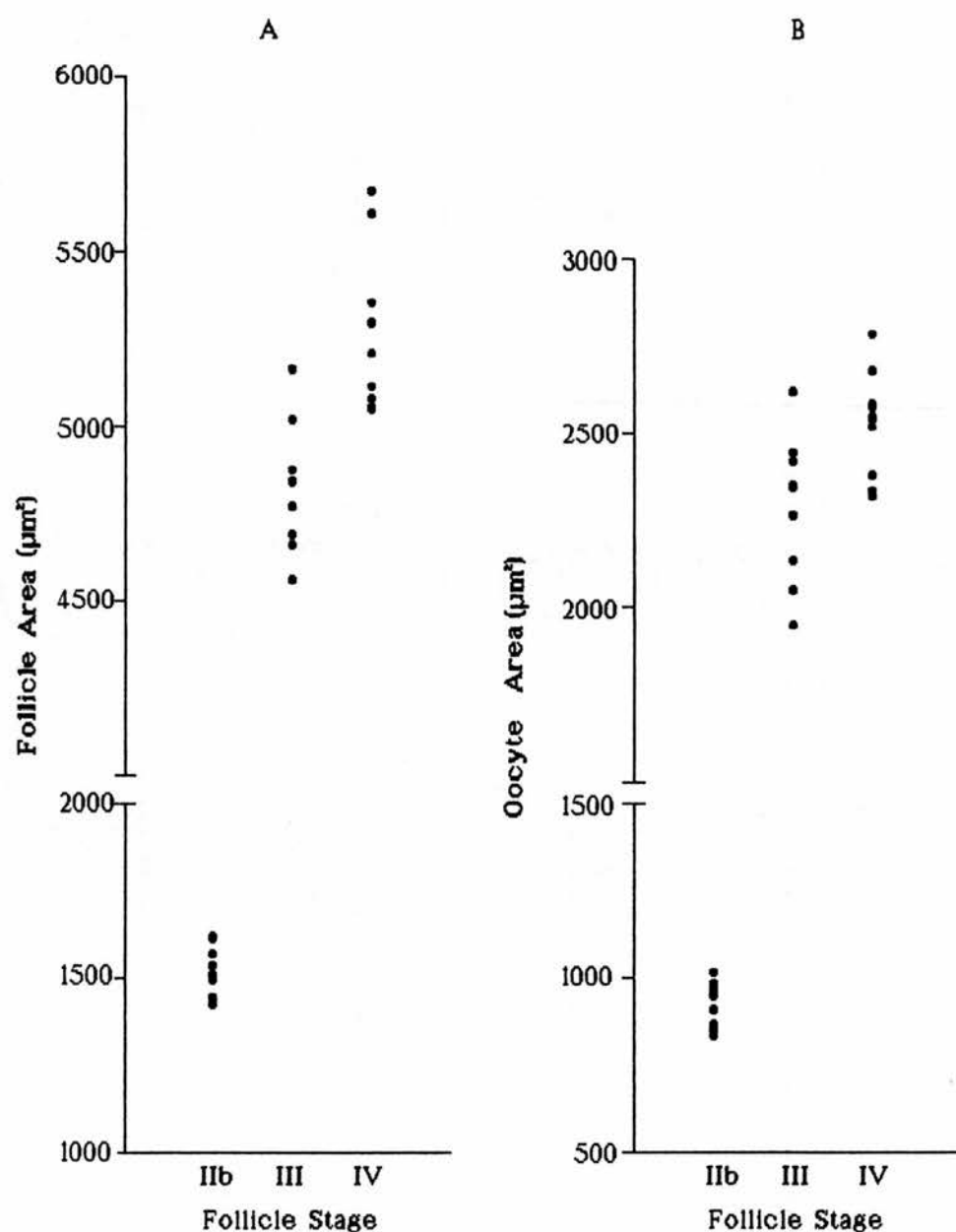


Figure 5.3. Reproducibility of measurements of follicle (A) and oocyte (B) area. Each point represents a repeat measurement for three stages of follicular development obtained by measuring a single follicle from each stage 10 times. The coefficient of variation ranges from 3-4.6%.

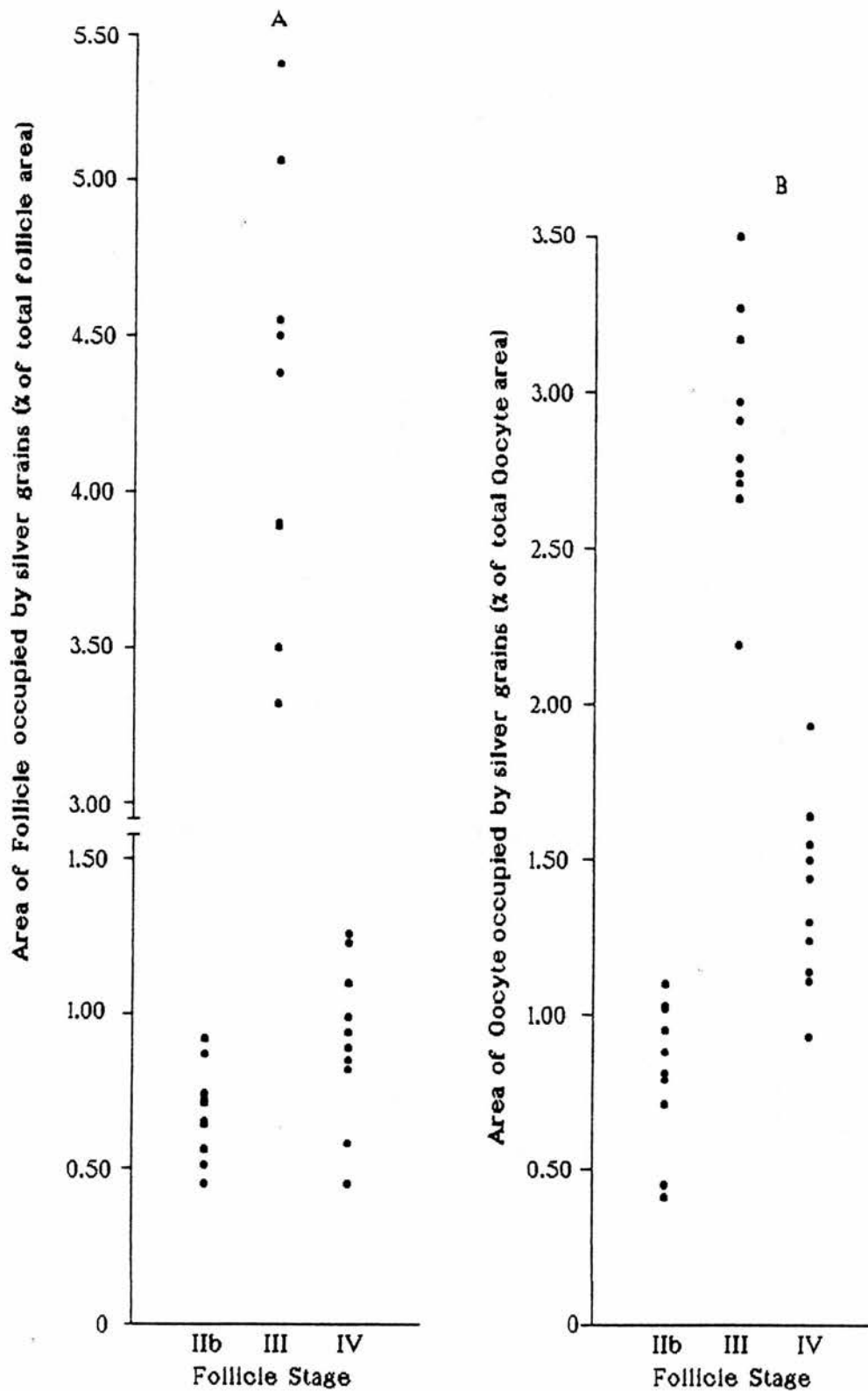


Figure 5.4. Reproducibility of measurements of follicle (A) and oocyte (B) incorporation of $[^3\text{H}]$ methionine as assessed by the % area occupied by silver grains. Each point represents a repeat measurement for three follicle stages obtained by measuring a single follicle 10 times. Coefficient of variation ranges from 12-29%.

Table 5.1. Background activity as measured over tissue-free regions of collagen gel for each of the experimental groups.

	<u>Area occupied by silver grains.</u>
	(μm^2)
Control	191.02 <u>±</u> 34.3
Oestradiol 17 β	112.01 <u>±</u> 6.9
FSH	169.57 <u>±</u> 25.3
FSH+Oestradiol 17 β	141.66 <u>±</u> 11.5

Values given are mean ± s.e.m, n = 40. Measurement frame area was 7736 μm^2 .

Table 5.2. The effect of hormone supplementation and stage on follicle area of follicles in collagen gel culture.

<u>Follicle</u> <u>Stage(1)</u>	<u>Hormone Treatment</u>			
	<u>Control</u>	<u>E₂</u>	<u>FSH</u>	<u>FSH+E₂</u>
Ila Follicle Area(μ^2)	1456.85 \pm 123.4 ^{a,b}	808.43 \pm 42.5 ^{a,c}	1648.79 \pm 131.6 ^{c,d}	1109.95 \pm 112.6 ^{b,d}
n	39	40	38	40
Ilb Follicle Area(μ^2)	2871.72 \pm 112.4 ^a	2496.85 \pm 93.8 ^{a,f,g}	2967.46 \pm 91.3 ^f	2704.98 \pm 81.7 ^g
n	39	39	37	40
III Follicle Area(μ^2)	4350.05 \pm 118.3	4095.38 \pm 117.4	3923.72 \pm 126.2	4140.95 \pm 138.7
n	40	40	36	39
IV Follicle Area(μ^2)	5140.90 \pm 152.2 ^h	4655.43 \pm 123.3 ^{h,i,j}	5415.47 \pm 176.7 ⁱ	5381.08 \pm 166.3 ^j
n	39	40	32	40

(1) Stage IV > III > IIb > IIa significantly different for all treatments
 Letters indicate pairs of means with a significant difference.
 Values are given as mean \pm s.e.m., p > 0.05.

from FSH but was significantly greater than E₂. There was no significant difference between E₂ and E₂+FSH treatments. Stage IIb follicle areas show no significant difference between control, FSH or E₂+FSH but a possible inhibitory effect of E₂ alone is seen. There was no significant difference between control, FSH or E₂+FSH treatment follicles but these had a significantly greater follicle area than the E₂ treated group. No effect was seen between the treatments on Stage III follicles, but the possible inhibitory effect of E₂ reached the significance level in Stage IV follicles. The E₂ group was significantly smaller than the other three treatments. FSH-treated follicles gave the largest mean follicle area at each stage except Stage III, but this difference in size did not reach significance at the $p = 0.05$ level. However FSH did reduce the inhibitory effects of E₂ when given in combination.

Table 5.3. summarises the data for oocyte area for each of the 4 treatments and follicle stages. In general the oocyte area of Stage IV follicles was equal to or greater than for Stage III. This is not surprising as the oocyte is near the end of its growth phase. Stage IIa follicles had the smallest oocyte area. However in the FSH group although the Stage IV follicles had the largest oocyte area the Stage IIb oocytes were significantly greater than Stage III. As can be seen in Table 5.3. there is no clear pattern for the effects of the treatments. Generally oestradiol appears to reduce oocyte size, there is little difference between control and FSH and combined treatment with oestradiol and FSH moderates the inhibitory effect of oestrogen. A notable exception are the Stage III follicles treated with FSH. Here a reduction in oocyte size compared with the other three treatments was noted. The zona pellucida makes a minor but significant contribution to oocyte diameter. However the light eosin stain used did not allow it to be easily discriminated in these preparations.

Table 5.3. The effect of hormone supplementation and stage on area of oocytes of follicles in collagen gel culture.

Follicle Stage	Hormone Treatment			
	Control (1)	E ₂ (2)	FSH (3)	FSH+E ₂ (4)
I Ia Oocyte Area (μ ²)	786.49±71.6abc	383.85±23.8ce	894.53±81.5ade	572.70±65.6bd
n	39	40	38	40
I Ib Oocyte Area (μ ²)	1487.72±54.7	1303.46±56.4f	1511.76±63.9f	1490.00±46.3
n	39	39	37	40
III Oocyte Area (μ ²)	1852.58±56.0g	1797.90±48.2h	1458.61±56.5ghi	1807.44±64.9i
n	40	40	36	39
IV Oocyte Area (μ ²)	1893.03±58.0	1716.18±54.3j	1781.78±60.0k	2006.50±75.4jk
n	39	40	32	40

(1) Control Stage IV not significantly different Stage III > Stage IIb > Stage I Ia.
 (2) Oestradiol Stage III not significantly different Stage IV > Stage IIb > Stage I Ia.
 (3) FSH Stage IV > Stage IIb not significantly different Stage III > Stage I Ia.
 (4) Oestradiol+FSH Stage IV > Stage III > Stage IIb > Stage I Ia.
 Letters indicate pairs of means with a significant difference.
 Values are given as mean± s.e.m., p > 0.05.

Membrana granulosa area follows the expected pattern with regard to follicle stage, Stage IV > III > IIb > IIa (Table 5.4). Again a consistently significant effect is not noted between the treatments for each stage but significantly reduced granulosa area for E₂ treated follicles of Stage IIa and IV compared with both control and FSH. No effect is seen for any treatment in Stage IIb follicles and FSH significantly reduces membrana granulosa area compared with the control group in Stage III follicles. Follicle counts were not carried out but the number of follicles in a gel was estimated in the range of 150-200. An assessment of follicle quality was not possible with the stain used. Indications of follicle atresia could not be distinguished with the stain used.

The task program measured the area of the membrana granulosa or oocyte occupied by silver grains and this measurement was expressed as a percentage of the total oocyte or membrana granulosa area. The effects of treatments on [³H]-methionine incorporation as assessed by the percentage of membrana granulosa and oocyte area occupied by silver grains are summarised in Tables 5.5. and 5.6. The results indicate little difference between methionine incorporation in both oocyte and membrana granulosa for control and FSH treatment but reduced incorporation in oestradiol treated follicles. However the data are variable and the results do not reach significance for the difference between FSH and control groups compared with E₂ and E₂+FSH. FSH and oestradiol together appeared to reduce methionine incorporation but to a lesser extent than oestradiol alone, but the difference does not reach significance at the 5% level. Although the $p = 0.05$ level was chosen for this study, where a significant difference existed between methionine incorporation by oestradiol-treated follicles and follicles of the same stage from the other treatment groups the p values lay in the range of 0.025-0.001, with the majority at the

Table 5.4. The effect of hormone supplementation and Stage on membrana granulosa area of follicles in collagen gel culture.

Follicle Stage(1)	Hormone Treatment			
	Control	E ₂	FSH	FSH+E ₂
IIa Granulosa Area(μ ²) n	662.05±54.3 ^a 39	424.58±20.8 ^{a,b} 40	754.26±55.2 ^b 38	537.25±51.3 40
IIb Granulosa Area(μ ²) n	1383.92±70.8 39	1193.38±48.4 39	1337.49±43.8 37	1214.97±51.0 40
III Granulosa Area(μ ²) n	2497.48±86.9 ^c 40	2297.48±91.1 40	2232.61±107.6 ^c 36	2333.51±90.8 39
IV Granulosa Area(μ ²) n	3247.87±125.6 ^d 39	2939.25±91.1 ^{d,e} 40	3420.78±152.3 ^e 32	3349.58±110.9 40

(1) Stage IV > III > IIb > IIa significantly different for all treatments.
 Letters denote pairs of means significantly different.
 Values are given as mean± s.e.m., p > 0.05.

Table 5.5. The effect of hormone supplementation on [³H]-methionine incorporation in the membrana granulosa of follicles in collagen gel culture as assessed by detected grain area.

<u>Hormone Treatment</u>					
<u>Control</u>		<u>E₂</u>	<u>FSH</u>	<u>FSH+E₂</u>	
<u>Follicle Stage</u>	<u>% of Area with detected grains</u>				
I Ia	Granulosa n	3.04±0.5 ^a _b 39	0.87±0.1 ^a _c 40	2.34±0.4 ^c 38	1.69±0.2 ^b 40
I Ib	Granulosa n	2.57±0.4 ^d _e 39	0.83±0.1 ^d _f 39	3.08±0.5 ^f _g 37	1.06±0.1 ^e _g 40
III	Granulosa n	3.93±0.7 ^h _i 40	1.10±0.1 ^h 40	1.73±0.2 ⁱ 36	2.10±0.4 ^j 39
IV	Granulosa n	3.34±0.6 ^k _l 39	1.02±0.2 ^k _m 39	3.77±0.9 ^m _n 32	1.96±0.4 ^l _n 40

Letters denote pairs of means with significant differences between treatments for each Stage.
 NB. Significant differences between treatments for different stages are not shown.
 Values are given as mean± s.e.m., p > 0.05.

Table 5.6. The effect of hormone supplementation on [³H]-methionine incorporation in the oocyte of follicles in collagen gel culture as assessed by detected grain area.

<u>Hormone Treatment</u>					
<u>Follicle Stage</u>	<u>% of area with detected grains</u>	<u>Control</u>			
			<u>E₂</u>	<u>FSH</u>	<u>FSH+E₂</u>
IIa	Oocyte n	2.05±0.4 ^a 39	1.04±0.1 40	1.77±0.3 38	0.85±0.1 ^a 40
IIb	Oocyte n	2.13±0.5 ^b 39	0.65±0.1 ^b _c 39	2.41±0.4 ^c _d 37	1.19±0.2 ^d 40
III	Oocyte n	3.26±0.7 ^e _f 40	0.86±0.1 ^e _g 40	2.38±0.3 ^g 36	1.42±0.3 ^f 39
IV	Oocyte n	2.45±0.5 ^h _i 39	0.93±0.2 ^h _j 39	3.07±0.6 ^j _k 32	1.15±0.2 ⁱ _k 40

Letters denote pairs of means with significant differences between treatments for each Stage.
 NB. Significant differences between treatments for different stages are not shown.
 Values are given as mean ± s.e.m., p > 0.05.

p = 0.001 level. The effect of oestradiol is impressive.

Discussion

This is the first study to quantitatively analyse protein synthesis by individual small follicles by image analysis and the results were encouraging. The differences detected between treatments indicated that small, preantral follicles maintained in collagen gel culture retain their ability to respond to hormonal treatments and provides evidence for the usefulness of the culture system in investigations of preantral follicle growth and differentiation. The combination of *in vitro* culture and computer-assisted image analysis represents a novel approach to the investigation of follicle development. The system used was able to detect hormonal effects on both simple area measurements and on the incorporation of radiolabelled methionine. The main problems and limitations encountered were with variability of the autoradiography and finding a suitable background stain. The problem of a higher density of grains at the edges of the slides might be solved by using stripping film to give a uniform thickness of photographic emulsion. Interference by background stain may be partially overcome by using dark field microscopy to visualise the silver grains and more flexible programs for autoradiography assessment are being developed for the system used.

The image analysis approach should prove particularly useful for measurements of follicle, membrana granulosa and oocyte area. Measurements of these parameters were relatively efficient and reliable even in the lightly stained tissues used in this study. The task files developed could be adapted to permit these measurements to be combined with a differential count of follicle stages and numbers as carried out in chapter 3 to

produce a potentially powerful method for studying follicle growth and atresia.

Combining the techniques used in the present study with differential counts may be an essential step. Follicle stages are defined by the number of granulosa cell layers and the size of the oocyte. However the size of granulosa cells does vary and follicle stage does not have to correspond to size, the heterogeneity of the stage II follicles which required the subdivision into stage IIa and IIb being a good example. It is possible that changes in granulosa cell size may occur due to a treatment but this would not necessarily force the follicle into a new stage. Another problem limiting the possible interpretation of the results is the lack of information on movement between the stages. For example an alternative interpretation of the effect of oestrogen on follicle size could be that the steroid had moved the larger follicles of a particular stage onto the next phase, leaving the smaller ones dominating that stage or in the case of FSH, the lack of a significant difference between the follicle areas for the control and FSH groups could reflect FSH-stimulated progression into the next phase. Alternatively treatments might promote atresia of larger follicles, again leaving smaller follicles to dominate. These problems of interpretation would be partially solved by including a differential count and using compartmental modelling.

The present study demonstrated that the expected effect of stage on total follicle area was maintained i.e. regardless of treatment later stage follicles had a greater area than those of an earlier stage. Hormonal influences on total follicle area were detected, control and FSH treated groups were essentially the same but oestrogen treatment resulted in a reduced follicle and membrana granulosa area. Concurrent treatment with FSH reduced the effect of oestradiol but this effect did not reach

significance for every follicle stage. The effect of E₂ appeared to extend to oocyte area and to the incorporation of tritiated methionine. These results differ from some reports of oestrogenic effects *in vitro* and *in vivo*. The use of serum in the culture medium may be a complicating factor, limiting the interpretation of the results (e.g Erickson, 1983). Treatment effects may be optimised by replacing the serum-supplemented medium with a defined medium. Serum contains a number of factors which could mask or inhibit treatment effects and complicate interpretation of the results. Serum might reduce the effects of steroids. Thanki & Channing (1979) reported a mitogenic response to E₂ by porcine granulosa cells in culture medium supplemented with 4% serum and indicate that this effect may be masked in 10% serum because the cells are already maximally stimulated.

In the rat oestrogen has been shown to have a stimulatory effect *in vivo* on large preantral follicles and the hypophysectomized or oestrogen-treated immature rat is a useful source of granulosa cells for *in vitro* studies. The granulosa cells obtained respond *in vitro* to FSH with increased oestrogen, progesterone and 20 α -dihydroprogesterone production (Wang et al., 1979). Oestrogen alone had no effect on the activity of primary cultures of rat granulosa cells but oestrogen-enhancement of FSH-stimulated aromatase activity has been demonstrated (Adashi & Hsueh, 1982; Zhuang et al., 1982).

Enhancement of LH-stimulated aromatase activity has also been reported (Zhuang et al., 1982). In the hamster FSH has been shown *in vivo* to cause follicle recruitment and DNA synthesis and *in vitro* to stimulate tritiated thymidine incorporation (Kim & Greenwald, 1984; Roy & Greenwald, 1986a, b; Roy & Greenwald, 1988). Oestrogen, however has not been shown to affect the growth of large preantral follicles in the hamster *in vivo* or *in*

vitro (Kim & Greenwald, 1984; 1987). Kim & Greenwald (1987) also investigated the effects of exogenous oestrogen on follicle growth in rabbit, mouse and guinea pig. In the guinea pig oestrogen increased ovarian weight and produced a significant increase in the numbers of antral follicles. In the rabbit oestrogens were found to stimulate the growth of small preantral follicles (2-5 layers of granulosa cells) but in the mouse larger preantral follicles (6+ layers) were affected.

In organ cultures of rat and mouse ovaries increased granulosa cell mitosis has been demonstrated after oestrogen treatment (Lostroch, 1957; Fainstat, 1968). Kent (1973) reported a variable response to oestrogen by immature mouse ovaries in organ culture and her results suggest that growth of 1-2 layered follicles was stimulated but growth of larger preantral follicles inhibited. Roy & Greenwald (1989) have developed a long-term culture system for preantral hamster follicles. In this system FSH was reported to increase labelling indexes, increase DNA content and reduce atresia. Oestradiol and progesterone however had no effects on DNA synthesis and after 24 hours oestradiol appeared to induce atresia. In the present study only follicles with up to 3 granulosa cell layers were measured and an oestrogen effect was clear on methionine incorporation although effects of area measurements require a more guarded interpretation. The effects of oestrogen reported in this and other studies do not demonstrate a direct effect on granulosa cell growth. Oestrogen effects are variable and could be mediated by other factors such as a growth factor.

A number of possibilities could account for the inhibitory effect of oestrogen *in vitro*. The mitogenic effects seen in some species *in vivo* might not be direct oestrogenic effects but linked to other paracrine mechanisms, possibly growth factors such as epidermal and fibroblast

growth factors (Kim & Greenwald, 1987). The dissociation and culture process could reduce the number of steroid receptors present, i.e cellular phenotype might be altered as happens in monolayer cultures. Cells from another steroid sensitive tissue, mouse vaginal epithelium, have reduced numbers of oestrogen receptors when dissociated by collagenase and cultured for 9 days but the same cells when transplanted with vaginal stroma give rise to normally cycling vaginal epithelium (Uchima et al., 1987, Cooke et al., 1986). Interestingly these cells *in vitro* respond to E₂ with a dose-dependent inhibition of proliferation, rather than the stimulation seen *in vivo* (Iguchi et al., 1987). The resumption of vaginal epithelium cyclicity after transplantation presents an interesting comparison with the growth of cultured follicles when transplanted under the kidney capsule as reported in the last chapter. More work is needed to establish whether the inhibitory effect noted is an artefact of the culture system, due to the lack of other co-factors or a true inhibitory effect.

The experiments described in this chapter indicate that the culture system when combined with image analysis can provide a useful approach to investigating small follicle development. Measurements of follicle and oocyte area need to be interpreted with caution in a compartmental system such as the ovary. Membrana granulosa area combined with estimates of individual granulosa cell area has potential as a method of estimating cell numbers. The measurement of tritiated methionine incorporation of the granulosa and oocyte compartments was more reliable. In this study incorporation was estimated as the percentage of the oocyte or granulosa area occupied by silver grains. The results suggest that protein synthesis is not proportionally any greater in the oocytes and membrana granulosa of stage IV or stage II follicles. The results suggest that with some development and improvement of the techniques the effects of hormones,

growth factors and ECM components on small follicle development could be studied *in vitro*.

CHAPTER 6

Preliminary experiments using a partially defined culture medium to explore the effects of epidermal growth factor on small follicular development.

Introduction

The role of local non-steroidal regulators in follicular physiology is currently an area of rapid expansion. The number of substances identified as having a possible paracrine role in the follicle is continually increasing and includes factors regulating oocyte maturation, regulators of luteinization, GnRH-like peptides, follicle regulatory proteins, gonadotrophin-binding inhibitors and the peptide growth factors such as epidermal growth factor and fibroblast growth factors. Other substances with a putative local role include neurotransmitters: the catecholamines, γ -aminobutyric acid (GABA) and vasoactive intestinal peptide (VIP). Sharpe (1984) has suggested three criteria for identifying a substance as an intragonadal hormone or factor: 1) evidence of intragonadal production, 2) the presence of receptors for the hormone within the gonad and 3) demonstrable biological action(s) within the gonad. The range of putative intragonadal hormones is summarised in Table 6.1: not all of these have, as yet, been shown to satisfy all three criteria. Some have still to be chemically characterized and the biological action of many has only been demonstrated *in vitro*. As in other areas of follicular physiology much of the focus of research into the growth factors has been on larger, antral follicles or on granulosa cells from the hypophysectomized, oestrogen-stimulated immature rat *in vitro*. General reviews of intraovarian hormones have been presented. Other ovarian hormones such as the steroids and inhibin are also thought to have important paracrine and autocrine functions. The role of the steroids has been discussed in earlier chapters.

Growth Factors and Follicular Function

The human primordial follicle has about 50 granulosa cells, the preovulatory follicle some 5×10^7 (Lintern-Moore et al., 1974): this

Table 6.1 Putative intraovarian regulators of follicle function.

	<u>Reference</u>
<u>Growth Factors</u>	
Epidermal growth factor (EGF)	May & Schomberg (1989)*
Fibroblast growth factor (FGF)	Gospodarowicz (1989)*
Transforming growth factor- β (TGF- β)	Carson et al. (1989)*
Insulin-like growth factor-I (IGF-I)	Carson et al. (1989)*
Platelet-derived growth factor (PDGF)	Tsafriri (1988)*
Erythroid Differentiation Factor (EDF)	Hasegawa et al., (1988)
<u>Oocyte Maturation Inhibitor</u>	Tsafriri (1988)*
<u>Follicle Regulatory Proteins</u>	Tonetta & diZerga (1986)*
<u>Neurotransmitters</u>	Tsafriri (1988)*
Vasoactive Intestinal Peptide	Ojeda et al., (1989)*
Catecholamines	Adashi & Hsueh (1981)
GABA	Erdo et al., (1985)
Adenosine	Billig et al., (1988)
<u>Gonadotrophin-binding inhibitors</u>	Ward et al., (1982)*
<u>Luteinization Factors</u>	Tsafriri (1988)*
<u>GnRH-like peptides</u>	Birnbaumer et al., (1985)
<u>Others</u>	
Interleukin-1	Fukuoka et al., (1988).

(* Denotes a review paper)

impressive expansion of the granulosa compartment represents a major proliferative effort. The role of classical hormones-such as the gonadotrophins and steroids- in this process has been discussed but there is some evidence to suggest that other mitogenic factors may also be involved. Experiments by May & Schomberg (1984) on granulosa cells *in vitro* suggest paracrine mechanisms may be important during granulosa proliferation and differentiation. The authors reported that increasing cell densities in culture enhanced LH receptor formation. The marked proliferation of the membrana granulosa and suggestions of paracrine and autocrine mechanisms focussed interest on the possible role of peptide growth factors. Granulosa-theca cooperativity is a recognised feature of the process of steroidogenesis but there is evidence for more extensive interactions between these cell types. McNatty et al. (1980) observed that coculture of human granulosa cells with thecal tissue increased granulosa cell proliferation. Extracts of thecal tissue or thecal cell conditioned medium have been shown to contain a protein factor able to stimulate proliferation in cultured granulosa cells (Makris et al., 1983). In a granulosa-theca coculture system which maintained physical separation of the cell types bovine thecal cells were found to promote granulosa cell proliferation. This effect was also found with theca-conditioned medium Bendell et al., 1988). Bendell and co-workers reported that the growth factor was not ether-extractable and appeared to be a peptide growth factor (or factors).

In this chapter the effect of EGF on small follicle function is explored using collagen gel culture of follicles in a serum-free medium. EGF effects are assessed by autoradiography and computer-assisted image analysis. Initial experiments assessing the serum-free medium and the main investigation into the effects of EGF are described.

Epidermal Growth Factor

EGF is a 53 amino acid, acidic, single polypeptide chain protein with a molecular weight of 6043 (Carpenter & Cohen, 1979). It was first isolated from the mouse submaxillary gland by Cohen (1962). It acts on target cells via specific, 170 kDa, membrane receptors (see Das, 1982 for a review of mechanisms of action) and has been shown to be mitogenic for a range of tissues. Early studies of EGF concentrated on a possible role in granulosa cell proliferation. EGF was first investigated for its proliferative effect on bovine granulosa cells *in vitro* by Gospodarowicz et al. (1977). A mitogenic effect was demonstrated and EGF also proved to be mitogenic for porcine, human and rabbit granulosa cells. EGF was not mitogenic for rat granulosa cells in culture (Gospodarowicz & Bialecki, 1979). EGF stimulation of granulosa cells proliferation *in vitro* was enhanced by the presence of serum suggesting synergistic actions with other growth factors. Investigations of porcine granulosa cell proliferation in culture suggested that EGF has a synergistic effect on proliferation with IGF-I and TGF- β (May et al., 1988).

Whereas EGF stimulates granulosa cell proliferation (except in the rat) it generally has an inhibitory action on granulosa cell differentiation. EGF was found to inhibit FSH-dependent stimulation of LH receptor induction in rat and to a lesser extent porcine granulosa cells in culture (Mondschein & Schomberg, 1981; Mondschein et al., 1981). EGF inhibits granulosa cell oestrogen production (Hsueh et al., 1981; 1984; Schomberg et al., 1983) and also LH-stimulated androgen production by rat thecal cells (Erickson & Case, 1983). Effects on progesterone biosynthesis are less clear. Using a range of 1-4 $\mu\text{g/ml}$ EGF an inhibitory effect on hen granulosa cells in short-term culture was demonstrated but the dose was 10 to 100-fold higher than used in other ovarian studies (Pulley & Marrone,

1986). An inhibitory effect was also seen in porcine granulosa cells *in vitro* (Schomberg et al., 1983). However granulosa cells from the oestrogen-stimulated hypophysectomized rat model responded to EGF with enhanced progesterone production (Jones et al., 1982; Knecht & Catt 1983). Trzeciak et al. (1987) have also demonstrated EGF stimulation of progesterone production in rat granulosa cell primary cultures.

In cultured porcine granulosa cells EGF enhanced iodinated FSH binding and other studies have revealed an interesting interaction between EGF and FSH (May et al., 1987). While EGF stimulates the FSH receptor content of porcine granulosa cells, treatment with FSH reduced the cells EGF binding: oestradiol and dihydrotestosterone had no effect (Buck & Schomberg 1988). Injection of FSH has been reported to reduce EGF binding capacity in homogenates of ovarian tissue from hypophysectomized rat (St Arnaud et al., 1983) but more recently a 2-3 fold increase in EGF-binding has been reported after FSH stimulation of granulosa cells, from diethystibestrol-implanted immature rats, over a 96 hour culture period (Feng et al., 1987). Buck & Schomberg (1988) estimated porcine granulosa cells from small follicles (1-3 mm) had about 18,000 EGF receptors per cell. Cells from larger, more differentiated follicles (6-8 mm) had approximately 10,000 receptors per cell. Rat granulosa cells have been estimated to have about 5,000 EGF receptors per cell (Jones et al., 1982, Feng et al., 1987).

Another *in vitro* action of EGF is the maturation of mouse and rat oocytes (Downs, 1989, Dekel & Sherizly, 1985). Dekel & Sherizly (1985) reported that EGF could induce maturation in the oocytes of cultured rat follicles. The effect was specific to EGF. Nerve growth factor and insulin failed to induce maturation. Downs (1989) tested a number of growth factors and hormones including TGF- β , FGF, PDGF and the IGFs, and found

that most had no effect on cumulus cell-enclosed murine oocyte maturation. FGF and insulin had a limited effect but EGF increased maturation by over 50% compared with controls. In an earlier study of denuded oocytes EGF did not stimulate maturation (Downs et al., 1988).

In the mouse ovary EGF synthesis has been confirmed by the detection of prepro-EGF by cDNA hybridization but localisation within particular ovarian compartments was not established (Rall et al., 1985). Skinner et al. (1987) demonstrated production of an EGF-like factor from immature rat thecal-interstitial tissue which inhibited radiolabelled-EGF binding. Granulosa cells did not secrete this factor. EGF-like activity has been detected in porcine follicular fluid, with small follicles having more than twice the level of activity of larger follicles (Hsu et al., 1987). In the human EGF has been identified in luteal cells of adult women and in the primordial follicles of the newborn child. (Khan-Dawood, 1987; Kasselberg et al., 1985).

EGF appears to satisfy 2 of the 3 criteria for an intragonadal hormone as suggested by Sharpe (1984): there is preliminary evidence for intragonadal production and the presence of receptors for the factor on ovarian cells is well established. However the biological actions described above have all been *in vitro*. An *in vivo* function has to be established to fulfil the third criterion. Established physiological roles for EGF *in vivo* are very limited. An inhibitory effect on ovarian development was reported after injection of EGF over the first 5 days *post partum* in mice (Lintern-Moore et al., 1981) but in rats, aged 10-13 days old, 5 days of EGF treatment increased follicular development (Gospodarowicz et al., 1978). In the mouse a physiological role in the maintenance of pregnancy is possible. Pregnant mice had their plasma EGF levels reduced by removal of the submandibular gland with a resulting 50% decrease in the number of

mice going to term. At present the evidence for the role of EGF in folliculogenesis is mainly derived from *in vitro* studies. A role in preantral follicle development is possible. Receptors for EGF appear to be more numerous in smaller follicles, but more research is needed in this area.

Gospodarowicz et al. (1977) demonstrated enhanced proliferation in response to EGF in serum-supplemented medium. They found that increasing the serum content of the culture medium enhanced the EGF responsiveness of bovine granulosa cells. Serum growth factors may be involved in this interaction. May et al., (1988) studied the effects of three growth factors identified in the ovary, IGF-I, TGF- β and EGF, on cultured porcine granulosa cell DNA synthesis and proliferation and reported synergistic effects. Given alone EGF resulted in a small but significant stimulation of [3 H]thymidine incorporation but IGF-I and TGF- β had little effect. Concurrent administration of all three growth factors markedly increased thymidine incorporation. The first step in this study was to develop a serum-free medium able to maintain follicles in collagen gel culture.

Preliminary experiments using defined culture medium to explore the effects of growth factors on small follicular development.

Materials and methods

Animals

The mice used in the following experiments were F₁ females aged 8-11 days post partum and derived from matings between CBA/Ca males and C57Bl/6 females. Breeding pairs were provided with a pelleted diet and water *ad libitum* and housed under a light period of 14 hours starting at

0700 GMT. Room temperature was maintained at 21 °C. The immature mice were used immediately after their removal from the mother. Adult F1 hybrid females were used as hosts for transplants. Hosts were bilaterally ovariectomized at six weeks of age and allowed to recover for at least 3 weeks before use. All operations were carried out under intraperitoneal tribromoethane anaesthesia (6.03g/Kg body weight).

Culture Media

All preparatory steps were carried out in HEPES-buffered medium M199 (Gibco) and follicles were cultured in bicarbonate-buffered M199 (Gibco) containing 10% donor calf serum or a serum substitute containing insulin, transferrin, selenium, linoleic acid and bovine serum albumin - CR-ITS+ (Flow Laboratories). ITS+ was used at the recommended level, 1 ml ITS+ per 100 ml medium to give final concentrations of the components in the culture medium of: insulin 6.25 µg/ml, transferrin 6.25 µg/ml, selenium 6.25 ng/ml, linoleic acid 5.35 µg/ml and bovine serum albumin 1.25 mg/ml. The serum had been heat-inactivated for 35 minutes at 55°C. All media were supplemented with gentamicin 100 µg/ml (Sigma), amphotericin B 2.5 µg/ml (Sigma) L- glutamine 100 µg/ml (Flow Laboratories) and sodium pyruvate 35 µg/ml (BDH).

Follicle isolation

Follicle isolation and collagen gel culture was carried out as described in chapter 3. All steps were carried out using ice-cold preparation medium, the bisected ovaries were incubated with gentle rotation at 37 °C in a 10ml tube containing Hepes-buffered medium with 1.5 mg/ml of collagenase (Sigma type 1) and 40 units/ml of DNase I (Sigma) for 30 minutes. After removal of the collagenase the follicles were freed by repeated pipetting. The isolated follicles were pelleted by gentle centrifugation, the supernatant removed and the follicle pellet resuspended

in 3 ml of medium and the follicles concentrated in the centre of the watchglass by gentle rotation. To increase the number of follicles per gel, 30 μ l samples were pipetted from the central concentration into the wells of a Terasaki plate (Flow Laboratories) and the rotation and pipetting was repeated until the required number of samples had been obtained. The follicles were set in collagen gel derived from rat tails. 25 μ l of ITS+ was substituted for serum when preparing the collagen gel solution.

Experiment 1 Comparison of ITS+ and donor calf serum supplemented medium.

Follicle isolation and collagen gel steps were carried out as described above. Seventeen mice were used and 9 gels containing ITS+ but not serum were formed. The follicles in gel were cultured in three wells, with 3 gels per well. Two wells received the usual 10% donor calf serum the third contained medium supplemented with ITS+. One of the serum wells was changed to the ITS medium at 4 days. The culture medium was replaced every 2 days. Single gels were harvested at intervals after 4-10 days in culture. The progress of the cultures was monitored by phase contrast microscopy. Gels were fixed in Bouin's fluid and processed for histology as before. Sections were cut at 7 μ m and stained with haematoxylin and eosin and mounted with DPX. Sections were viewed at x100 and x400 magnification.

Experiment 2 *In vivo* assessment of follicles after 5 days in culture in ITS+ supplemented medium.

Follicle isolation and collagen gel steps were carried out as described above. The ovaries from 15 mice were used and 6 follicle-loaded gels formed. The gels were cultured for five days in ITS+ supplemented

medium. After five days in culture the gels were transferred under the kidney capsule of ovariectomized F1 hosts as previously described. Hosts were monitored daily from day 10 after transplantation for vaginal opening and vaginal smears obtained by lavage and stained with Giemsa. The mice were sacrificed by cervical dislocation, one mouse on day 10 after transplantation of the follicles, the remaining 5 on day 14. The grafts were dissected free from the kidney and fixed in Bouin's fluid for histology. At autopsy the animals were checked to ensure the ovariectomy had been complete and that no ovarian fragments were present, the uterus was removed, cleared of attached tissues and the wet weight obtained. Sections were cut at 7 μ m and stained with haematoxylin and eosin. Sections were viewed at x100 and x400 magnification.

Experiment 3 The effect of EGF on follicular growth *in vitro*.

Follicles were isolated from the ovaries of 24 immature mice as described above. No serum was added to the collagen gel solution. Twelve gels were formed and cultured in medium supplemented with donor calf serum for 4 days with the medium replaced at 48 hours. On the fourth day the serum supplemented medium was removed and replaced with ITS+ supplemented medium. Two wells contained ITS+ only medium and served as controls. EGF (Sigma) was added to the other 2 wells at a final concentration of 10 ng/ml. After 24 hours [3 H]thymidine (Amersham International, specific activity 85Ci/mmol) was added to one control and one EGF well to give a concentration of 5 μ Ci/ml. On day 6, three hours before the termination of the cultures, the medium was removed from the remaining control and EGF wells and the gels well washed twice with methionine-free medium 199 (Gibco). Then 0.25ml per gel of $\frac{1}{4}$ -strength methionine medium supplemented with ITS+ and, where appropriate, EGF containing 5 μ Ci/ml 3 H-Methionine

(Amersham International, specific activity 85Ci/mmol) was added. The follicles were returned to the incubator and cultured for three hours. At the end of the culture period the radioactive medium was removed from both thymidine and methionine treated wells and the gels washed twice with normal culture medium. The gels were then fixed in Bouin's fluid and processed for autoradiography. A duplicate experiment was performed.

Autoradiography and Image Analysis

After 24 hours in Bouin's fluid the gels were dehydrated and embedded in paraffin wax (see appendix). Fresh alcohols and wax were used at each step. 5 µm sections were cut and the sections were brought down to water through an alcohol gradient. Free thymidine was removed at this stage by immersing the slides in 1% perchloric acid at 4°C. In the darkroom the slides were coated with Kodak K2 Nuclear Emulsion. After exposure at 4°C in light-tight boxes for one week the slides were developed in Kodak D19 developer and fixed with Ilford Hypam Fixer. Thymidine and methionine groups were handled separately but to minimise variation due to autoradiographic processing duplicate experiments were dipped and processed for autoradiography together. Sections were lightly stained with eosin and mounted with DPX.

Image analysis was carried out using the system and task files described in the last chapter. The same parameters, follicle area, membrana granulosa area, oocyte area, membrana granulosa grain area and oocyte grain area were subtracted from the data files and used for analysis. A check on the background activity in tissue-free areas of collagen gel were also carried out.

Analysis

Data was analysed using the SPSS/PC+ statistical analysis program

(Chicago, Illinois, USA). Descriptive statistic and two way analysis of variance were calculated for the follicle measurements. One-way analysis of variance was used to assess the data from measurement of background activity. Multiple comparisons were carried out using Tukey's test with unequal sample size as described by Zar (1984). The chosen level of significance was $p = 0.05$.

Results

Assessment of the histological sections for experiment 1 did not reveal any gross morphological difference between follicles grown in ITS+ or serum supplemented medium. Follicles ranged from primordial to large multilaminar stages and no antra or theca were seen. After 10 days in culture the ITS+ follicles appeared to be the same as those in the serum control group. Transplantation of follicles after 5 days in ITS+ culture resulted in oestrogenisation of the vaginal smears: vaginal opening was established by Day 10 in all mice. Table 6.2 summarises the results of the vaginal smears and uterine weights. The mean uterine weight was 0.057 g (mean uterine weight for the collagen gel only control transplants reported in chapter 4 was 0.032 g). Autopsy reveal that ovariectomy was complete; no ovarian fragments were present which might account for the oestrogenisation. Histology of the grafts revealed a range of normal looking follicles of all stages, including large antral follicles. Antral formation and theca layers appeared normal but oocytes degenerated within the large follicles. There was no evidence of ovulation.

Experiment 3 did not produce any results for [^3H]thymidine incorporation. When developed, the autoradiographs displayed a high

Table 6.2. The pattern of vaginal smears produced by transplantation of follicles in a collagen gel matrix after culture for five days in ITS+ supplemented medium

Mouse No.	Days after Transplant	Leucocytes	Nucleated Epithelial Cells	Cornified Epithelial Cells	Mucus Uterine Wt(g)
1	10	no smears			0.053
2	11	++	+	+	0.032
	12	+		++	
	13		++	+	
	14			++	
3	11	+	+	+	0.068
	12			+++	
	13	+		+	
	14			++	
4	11	+++		+	0.023
	12	+		+	
	13	++	+	+	
	14	+	+	++	
5	11		++	+	0.071
	12		++	+	
	13		+	+++	
	14	+		+++	
6	11		+	+++	0.096
	12		+	++	
	13			+++	
	14	++	+	+	

Mean uterine weight = 0.057 g.

density of silver grains both in the tissues and in tissue-free regions of the collagen gel. The density of grains was also judged too high for accurate measurement using the image analysis system. The cultures incubated with [^3H]methionine produced measurable results. One-way analysis of variance of the data for background activity in tissue-free regions of collagen gel revealed no significant differences between the mean grain area of the 2 treatments.

Two-way analysis of variance of the effects of treatment and follicle stage on each of the 5 parameters produced variable results. As expected follicle stage produced a significant effect on follicle, membrana granulosa and oocyte areas. It had no effect on the area of the membrana granulosa occupied by silver grains. A significant effect was produced on the area of the oocyte occupied by grains. A significant interaction between Stage and treatment only occurred for the oocyte grain area. As can be seen from Table 6.3 all three area measurements follow the pattern: Stage IV > III > IIb > IIa. As there was no effect of EGF compared with control and the stage effect followed the expected pattern, multiple comparison testing was not used.

Table 6.4 summarises the data for grain-area measurements of both membrana granulosa and oocyte. Treatment with EGF produced a significant effect on the area of the membrana granulosa occupied by silver grains but had no effect on the oocyte. Follicle stage had no effect on membrana granulosa grain-area but did influence oocyte grain-area. Tukey's test was carried out to identify the sources of significance.

Figure 6.1 shows an EGF-treated Stage III follicle. An interesting feature of the follicle is the presence of a few spindle shaped cells at the edge of the follicle. These were occasionally seen and might represent early theca differentiation.

Table 6.3 The effect of EGF or control treatment and stage on follicle, oocyte and membrana granulosa area of follicles in collagen gel culture.

<u>Follicle</u> <u>Stage (1)</u>		<u>Treatment</u>			
		<u>Control</u> (μm^2)	<u>n</u>	<u>EGF</u> (μm^2)	<u>n</u>

IIa	Follicle Area	1675.97+79.9		1761.45+93.9	
	Oocyte Area	810.87+45.7	(39)	818.25+45.5	(40)
	Membrana granulosa Area	865.15+41.7		943.20+60.9	

IIb	Follicle Area	2914.87+112.8		3254.10+102.2	
	Oocyte Area	1429.10+59.1	(39)	1506.60+48.3	(40)
	Membrana granulosa Area	1485.77+75.2		1747.25+68.2	

III	Follicle Area	4477.44+108.3		4406.44+98.7	
	Oocyte Area	1689.15+54.2	(39)	1528.92+51.6	(39)
	Membrana granulosa Area	2788.28+87.4		2877.51+93.3	

IV	Follicle Area	6425.05+179.8		6287.19+152.5	
	Oocyte Area	1825.70+56.7	(40)	1780.00+56.4	(32)
	Membrana granulosa Area	4599.35+166.0		4507.19+132.6	

Values are mean \pm s.e.m., n = the number of follicles measured

(1) Stage but not treatment produced a significant difference in two-way ANOVA. Stage IV > III > IIb > IIa for both treatments. Multiple comparison testing not carried out. p = 0.05.

Table 6.4. The effect of EGF or control treatments on [³H]-methionine incorporation in the oocyte and membrana granulosa of follicles in collagen gel culture as assessed by detected grain area.

		<u>Treatment (2)</u>			
<u>Follicle</u>	<u>% of Area with</u>	<u>Control</u>	<u>n</u>	<u>EGF</u>	<u>n</u>
<u>Stage (1,3)</u>	<u>detected grains</u>				

I Ia	Membrana granulosa Oocyte	3.88±0.4 ^{a b} 3.06±0.3 ^d	(39)	6.16±0.4 4.35±0.3 ^e	(40)

I Ib	Membrana granulosa Oocyte	5.43±0.3 4.08±0.2 ^f	(39)	6.23±0.4 5.09±0.4	(40)

III	Membrana granulosa Oocyte	5.29±0.3 3.41±0.2 ^g	(39)	6.31±0.4 ^b 4.27±0.3 ^h	(39)

IV	Membrana granulosa Oocyte	4.86±0.2 ^c 6.86±0.7 ^{d e f g h}	(40)	7.68±0.6 ^{a c} 5.14±0.5	(32)

Values are mean ± s.e.m., n = the number of follicles measured.
 (1,2) Stage had no effect on membrana granulosa measurement but treatment had a significant effect using two-way ANOVA.
 (3) Stage had a significant effect on oocyte grain measurements but there was no effect from treatment.
 Letters denote pairs of means with significant differences between them as determined by multiple comparison testing. $p > 0.05$.

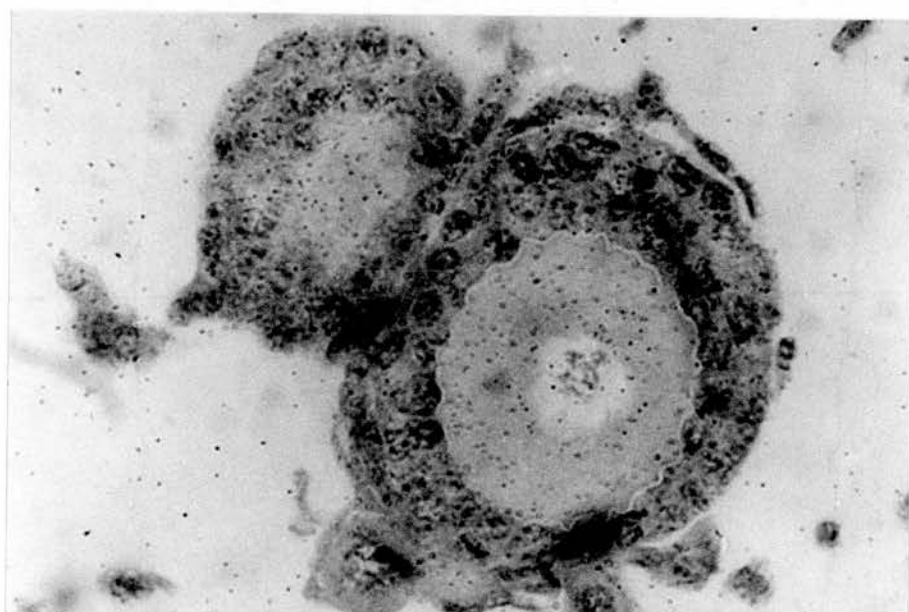


Figure 6.1. A section of collagen gel after culture in EGF-supplemented medium. A few spindle-shaped cells which might be a proto-theca can be seen. Autoradiograph with a light eosin stain (x480).

Discussion

The results from this study indicate that the serum-free medium is suitable for maintenance of follicles in collagen gel culture and that at least some of the follicles retain their developmental potential and can progress *in vivo* to the Graafian stage after transplantation to ovariectomized hosts. The results with thymidine were disappointing but the high background activity obtained could be overcome with more developmental work to identify the source of this technical problem. The methionine results are a little more encouraging. EGF treatment produced no effect on the area of the follicle, oocyte or membrana granulosa but methionine incorporation was significantly greater in EGF-treated Stage IV follicles compared with controls. However variability in the grain area measurements was a problem and the results need to be interpreted with caution. An interesting contrast was noted between the oocyte and granulosa grain measurements. Follicle stage was not a significant factor in membrana granulosa incorporation of methionine but was significant for the oocyte. The oocyte did not respond to treatment but stage was significant. This might be interpreted as showing an effect on the oocyte mediated through the granulosa cells although no significant difference was noted between control or EGF treated oocytes from the same stage of follicle.

In Stage IIa and IIb EGF-treated follicles- follicle, oocyte and membrana granulosa area was greater than in controls but the difference did not reach significance at the $p = 0.05$ level. Granulosa and oocyte incorporation of labelled methionine was greater in EGF than control follicles and oocytes of all stages but again fails to reach significance. The experiments need to be repeated with perhaps improved

autoradiography before definite conclusions can be reached. EGF is known to interact with serum factors and the culture medium used in these experiments was very basic. Further work is required in defining a suitable basal medium. FSH might be an essential component (Qvist et al., 1990). The possible factors which might interact with EGF include FSH, TGF- β , FGF, and the IGFs.

TGF- β is a peptide growth factor secreted by both ovarian thecal and granulosa cells in culture (Skinner et al 1987; Bendell & Dorrington, 1988; Kim & Schomberg, 1989). A number of studies indicate that TGF- β may enhance rat granulosa cell differentiation. Enhanced FSH-stimulated LH receptor induction and increased inhibin production have been reported (Knecht et al., 1986, Dodson & Schomberg, 1987; Zhang et al., 1988). TGF- β has been shown to stimulate activity in murine granulosa but not theca cells (Adashi et al., 1989). The proliferative effects of TGF- β on granulosa cells are more variable and interactions with other growth factors may be important. TGF- β with FSH promoted DNA synthesis in immature rat granulosa cells and when combined with EGF and IGF-I promoted DNA synthesis in granulosa cells from small porcine follicles (Dorrington et al., 1988; May & Schomberg, 1988). In bovine granulosa cells TGF- β has an inhibitory effect on proliferation (Skinner et al., 1987). An additional TGF- β effect reported is stimulation of meiotic maturation in oocytes from PMSG-treated immature rats (Feng et al., 1988).

Ovarian granulosa and possibly luteal cells produce another peptide growth factor, basic FGF (Neufeld et al., 1987). bFGF is an angiogenic factor but has also been shown to be mitogenic for bovine granulosa cells *in vitro* (Gospodarowicz et al 1977). There is also evidence that bFGF inhibits differentiation of bovine granulosa cells in culture and it could therefore have a role in preantral follicle development. Rat granulosa cell

proliferation is not stimulated by bFGF, it inhibits LH receptor induction by FSH and stimulates progesterone synthesis (Mondschein & Schomberg, 1981; Baird & Hsueh, 1986). IGF-I is produced by granulosa cells and its production is regulated by FSH and oestradiol-17 β (Hsu & Hammond, 1987a) and growth hormone (Davoren & Hsueh, 1986; Hsu & Hammond, 1987b). IGF-I is mitogenic for bovine and porcine granulosa cells but no mitotic effects were evident when rat granulosa cells were treated *in vitro* (Savion et al., 1981; Baranao & Hammond, 1984). IGF-I modulates a number of differentiated functions of granulosa cells including progesterone biosynthesis (Veldhuis et al., 1986; Adashi et al., 1984a), LH receptor induction (Adashi et al., 1984b), oestrogen biosynthesis (Adashi et al., 1985) and proteoglycan biosynthesis (Adashi et al., 1986).

An important role for peptide growth factors in follicular development seems likely but direct *in vivo* evidence is still lacking. *In vitro* studies of growth factor actions indicate regulation of both proliferation and differentiation of granulosa cells with additional actions on the theca. However the exact role of individual growth factors is difficult to determine. Interactions between the growth factors and other intragonadal and systemic regulators of follicular function appear complex. In particular there is a lack of information on the role of growth factors on preantral development. The complexity of growth factor interactions indicates that an *in vitro* approach in defined conditions may be essential. The results of this present study were inconclusive but suggest that the techniques employed could contribute to the investigation of the effects of growth factors on preantral follicle development.

APPENDIX 1

Fixation and Staining

Appendix 1 - Fixation and Processing for Histology

Fixatives.

Bouin's Fluid

Saturated aqueous picric acid	75 ml
Formalin	25 ml
Acetic acid	5 ml

Tissues fixed overnight, dehydrated in alcohol (range 70% - 95%, then absolute alcohol). Tissue was cleared in xylene, infiltrated by immersion in several changes of paraffin wax and finally embedded in paraffin wax in a cuboidal mould. Sections cut at 5 or 7 μ m. For autoradiography fresh solutions were used at each step.

Formalin

10% formalin in tap water

Formol calcium

Formalin	10 ml
Calcium chloride	1 g
Water	to 100 ml

Tissues fixed in formalin for 1 hour and then overnight in formol calcium. Used for study of blood vessels and theca in grafted follicles (Chapter 4).

Haematoxylin and Eosin

- 1 Dewax in xylene - 15 minutes
- 2 Hydrate in alcohol absolute - 90% - 70%
- 3 Wash in water
- 4 Haematoxylin 5 minutes
- 5 Rinse
- 6 3 dips in acid alcohol
- 7 Rinse
- 8 Scott's tap water substitute to blue for 3 minutes
- 9 Wash for 3 minutes
- 10 Counter stain with eosin 2 minutes
- 11 Rinse in water (3 dips)
- 12 Potassium alum 3 minutes
- 13 Wash
- 14 Dehydrate in alcohol 70%-90%-absolute
- 15 Clear in xylene
- 16 Mount with DPX and coverslip

Light eosin stain for autoradiography.

- 1 Dewax in xylene - 15 minutes
- 2 Hydrate in alcohol absolute - 90% - 70%
- 3 Wash in water
- 4 2 dips in eosin
- 5 2 dips in potassium alum
- 6 Dehydrate and mount as above.

'TRIS' Buffer (for DAB)

0.2M TRIS (hydroxymethyl) aminomethane (CH_2OH)₃CNH (MW 121).
Dissolve 24.2 g in distilled water and make up to 1 L.

0.1 N hydrochloric acid (MW 36.5) about 8.5 ml concentrated HCl/L

0.2M tris	25 ml
0.1N HCl	40 ml
distilled water	35 ml
pH 7.5	

Diaminobenzidine (DAB) Method for Peroxidase Staining

Preparation

Make up a saturated solution of 3:3 diaminobenzidine tetrahydrochloride in 10 ml of TRIS buffer, pH 7.6, and add 0.1 ml 1% hydrogen peroxide.

Technique

- 1 Rinse sections in distilled water.
- 2 Place in incubating medium for 5 minutes at room temperature.
- 3 Wash in 3 changes of distilled water.
- 4 Counterstain in 2% methyl green for 5 minutes. Wash in distilled water.
- 5 Dehydrate in alcohols, clear in xylene and mount in synthetic resin medium (DPX).

Results: sites of peroxidase activity - fine brownish granules, nuclei green.

APPENDIX 2

Published Material

Published Material

Torrance C, Telfer E, Gosden RG (1987) The isolation and culture of mouse ovarian follicles. A videotape programme presented to the Society for the Study of Fertility, Annual Summer Meeting, York.

Torrance C, Telfer E, Gosden RG (1988) An investigation of mouse ovarian follicles in collagen gel culture. *J. Reprod. Fert. Abstr. Series 1*, p24, abstr.

Torrance C, Telfer E, Gosden RG (1989) Quantitative study of the development of isolated mouse pre-antral follicles in collagen gel culture. *J. Reprod. Fert.* 87:367-374.

Telfer E, Torrance C, Gosden RG (1990) Morphological study of cultured preantral ovarian follicles of mice after transplantation under the kidney capsule. *J. Reprod. Fert.* 89:565-571.

Gosden RG, Hunter RHF, Telfer E, Torrance C, Brown N (1988) Physiological factors underlying the formation of ovarian follicular fluid. *J. Reprod. Fert.* 82:812-825.

Quantitative study of the development of isolated mouse pre-antral follicles in collagen gel culture

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Summary. Follicles were isolated from the ovaries of 10-day-old C57BL6/CBA F₁ hybrids by mechanical and enzymic treatment, embedded in a collagen-gel matrix to maintain the 3 dimensional integrity of the follicle and cultured for up to 14 days. Gels were removed at various times during the culture period and prepared for histology. Follicles grew from unilaminar to multilaminar stages within 6 days of the culture period. A more detailed assessment of growth by counting follicles at different stages and measuring oocyte and follicle diameters showed that follicle growth was maintained for up to 14 days in culture. Initially the proportion of unhealthy follicles was high but this declined after 6 days in culture.

Keywords: pre-antral follicles; collagen gel; culture; mouse

Introduction

The follicle is the functional unit of the mammalian ovary and has a dual role—it provides the micro-environment for oocyte growth and maturation and is responsible for sex steroid production. The ovary of an adult mouse contains thousands of follicles at various stages of development. There are three basic types of follicle: (1) non-growing or 'primordial' follicles, (2) preantral follicles in which oocyte growth and increase in granulosa cell numbers and layers is occurring, and (3) antral follicles which contain a fully grown oocyte with granulosa cells and a layer of differentiated theca cells. Primordial follicles move continually from the non-growing pool to enter a growth phase.

Follicles progress towards the antral stage without a further resting stage: a few are ovulated, the majority of follicles are lost through atresia. Little is known of the factors initiating and controlling growth of small follicles and extrapolation from the detailed knowledge of antral follicles may be unreliable, although a useful starting point. Recently, attention has concentrated on the role of paracrine and autocrine mechanisms in controlling ovarian function (reviewed by Hsueh, 1986). Various studies, mostly conducted *in vitro* using monolayers of granulosa cells, have indicated effects on growth and maturation of ovarian steroids (androgens, oestrogens and progesterone), and peptides (GnRH-like peptides, EGF, TGF- β , insulin, insulin-like growth factor-I (IGF-I), vasoactive intestinal peptide (VIP)) and even neurotransmitter substances. Most of these studies involved granulosa monolayers as the model, yet it is not clear how closely the state of differentiation reflects that *in vivo*. Evidence has been obtained that the secretory activity of granulosa cells is altered by cytoskeletal inhibitors and by culture in collagen gel, both of which are expected to affect the shape of the cell (Carnegie & Tsang, 1988).

To mimic physiological conditions it is preferable to study follicle growth and metabolism in isolated intact follicles in which normal three-dimensional relationships are maintained between

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granulosa cells and the oocyte. Furthermore, separation of the granulosa-oocyte unit from the stroma/theca cells at the basement membrane interface is a potentially valuable method for investigating cellular interactions. In this study a technique has been developed to isolate and culture intact follicles in a collagen-gel matrix.

Materials and Methods

Animals. The mice used in this study were F₁ females aged 8–11 days *post partum* and derived from matings between CBA/Ca males and C57BL/6 females.

Culture media. All preparatory steps were carried out in Hepes-buffered medium M199 (Gibco, Paisley, UK) and follicles were cultured in bicarbonate-buffered M199 (Gibco). Both media were supplemented with gentamicin (Sigma, Poole, Dorset, UK), amphotericin B (Sigma), L-glutamine (Flow Laboratories, Irvine, UK) and sodium pyruvate (BDH, Poole, Dorset, UK). The culture media also contained 10% donor calf serum (Flow Laboratories) which was found by bioassay to contain undetectable amounts of gonadotrophins (C. Torrance, unpublished). Sera were heat-inactivated at 55°C for 35 min. Preparation media had an osmolality of 290–310 mosmol kg⁻¹ as measured by a Wescor vapour pressure osmometer and a pH of 7.4. The culture media had an osmolality within a range of 280–300 mosmol kg⁻¹.

Collagen-gel solution. Collagen gel was extracted from the tail tendons of rats by the methods of Ehrmann & Gey (1956) and Chambard *et al.* (1981). The tendons were transferred to 70% alcohol, and rinsed in sterile distilled water. Then 1 g of tendon was added to 100 ml 1:1000 acetic acid and stirred at 4°C for 48 h. The solution was centrifuged at 2000 g on a benchtop centrifuge for 1 h. The solution was stored at 4°C for up to 8 weeks. Gels were prepared immediately before use by mixing 200 µl serum with 200 µl concentrated (×10) Medium 199 at 4°C. The pH was adjusted by adding 500 mM-NaOH until the indicator (phenol red) turned the appropriate colour for physiological pH.

Follicle isolation. All steps were carried out using cold preparation media and in each experiment 24 mice were used. The animals were killed by decapitation and the ovaries were removed and placed in a watchglass containing medium. All subsequent steps were carried out using sterile techniques in a laminar flow hood. The ovaries were bisected with fine needles. The bisected ovaries were incubated with gentle rotation at 37°C in a 10 ml tube containing medium with 1.5 mg collagenase/ml (Sigma, Type 1) and 40 U DNase I/ml (Sigma). After 30 min the ovaries were centrifuged (100–200 g) the tissue and the supernatant was removed. The pellet was resuspended in 10 ml medium and centrifugation repeated. The follicles were resuspended in 3 ml medium and transferred to a watchglass.

Under a dissecting microscope follicles were isolated by repeated pipetting of the bisected ovaries using Gilson pipettes. As the fragments decreased in size pipette tips of progressively smaller diameter were used. To minimize mechanical trauma from repeated pipetting, freed follicles were harvested frequently and filtered through a 125 µm nylon mesh (Nybolt ASTM 120-125, Simon Ltd, Stockport, Cheshire, UK) to remove lumps. The filter was flushed to remove trapped follicles. The follicles were pelleted by gentle centrifugation, the liquid was removed and the follicle pellet was resuspended in 3 ml medium.

The pellet was transferred to a watchglass and gently pipetted to break up clumps. Gentle rotation concentrated the follicles in the centre of the watchglass. Then 20 µl samples were pipetted from the central concentration into the wells of a Terasaki plate (Flow Laboratories). The rotation was repeated until 16 samples had been obtained. A Kleenex tissue was used to remove excess fluid from the wells and 10 µl of the collagen-gel solution were added and pipetted once to suspend the follicles. The follicles were incubated for 2–3 min at 37°C until the gel had set. After 20 µl gel solution were pipetted into empty wells, the 10 µl set gels were transferred to them. The double gels were allowed to set as before. Double gelling was necessary to avoid follicle losses resulting from collagen gel contraction during culture and processing for histology. Four gels were transferred to the wells of a Linbro tissue-culture plate containing 2 ml culture medium. The gels were incubated at 37°C with 5% CO₂ in a humidified incubator. The medium was changed 24 h later and every 3rd day.

Follicles could be monitored during culture by using inverted phase-contrast microscopy because collagen gel is transparent. At the termination of culture the gels were fixed overnight in aqueous Bouin's fluid, embedded in paraffin wax and sectioned at 5 µm. Sections were stained with haematoxylin and eosin and mounted with DPX.

Classification of follicles. Follicles were observed under ×400 magnification and classified according to the number of cell layers surrounding the oocyte, based on the established system of Mandl & Zuckerman (1951): Stage I, oocyte surrounded by a single layer of squamous granulosa cells; Stage II, growing oocyte surrounded by a single layer of cuboidal granulosa cells; Stage III, growing oocyte surrounded by 2 layers of cuboidal granulosa cells; Stage IV, growing oocyte surrounded by 3 layers of cuboidal granulosa cells; Stage V, oocyte surrounded by 4 or more layers of cuboidal granulosa cells but no antrum.

Follicles were also classed as disrupted (including free oocytes and any follicles with a disrupted granulosa layer) or intact (granulosa layer(s) intact). The quality of the intact follicles was also assessed. Follicles were classed as intact but damaged if they had more than slight contraction of the oocyte from the granulosa layers, with nuclear contraction, with obvious separation of the granulosa layers, or with more pycnotic cells than cell layers.

Longitudinal study of follicle growth. Experiments were designed to establish the pattern of follicle growth in collagen-gel culture. For each experiment 4 gels from one well were allowed to equilibrate in the incubator for 1 h and then harvested for histology to obtain information on the numbers, distribution and quality of follicles in the gels at the beginning of the culture period (0 h). Groups of 4 gels from the same well were harvested at intervals during the culture period (see Fig. 1) so that an impression of the pattern of growth over a 14-day culture period would be gained. The results from 11 disaggregation experiments and more than 100 gels are reported. Differential follicle counts using the above classification system were carried out on all gels.

Every section was counted in the 0-h gels and in gels cultured for 6 or more days in which numbers were lower. Every 3rd section was counted for gels from 1 to 5 days in culture. Follicles were counted only when the follicle section contained the nucleolus of the oocyte. Nucleolar diameters were measured in 22 follicles for Stage I, II and III follicles in 0-h gels using an image-shearing micromasurement system (Vickers Instruments, York, UK) under $\times 100$ oil immersion optics. These measurements were used to estimate a correction factor to compensate for overcounting resulting from the possibility of the nucleolar marker being present in more than one section. Total numbers of follicles were calculated by multiplying the number counted by 3 (the sampling frequency) and the correction factor. As Stage V follicles were absent in the 0-h gels, the Stage IV correction factor was used. Stage I (primordial) follicles were present in the gels but were not counted.

Results

Figure 1 summarizes the data of the follicle counts for individual gels over the course of the culture. The mean number of follicles declined with time. The proportion of follicles intact but showing some histological evidence of damage ranged from 33 to 66% between Days 2 and 5. On Days 6–8 the percentages of these follicles decreased to 22, 28 and 17%, and increased after Day 9 to 40%, reaching 64% by Day 14.

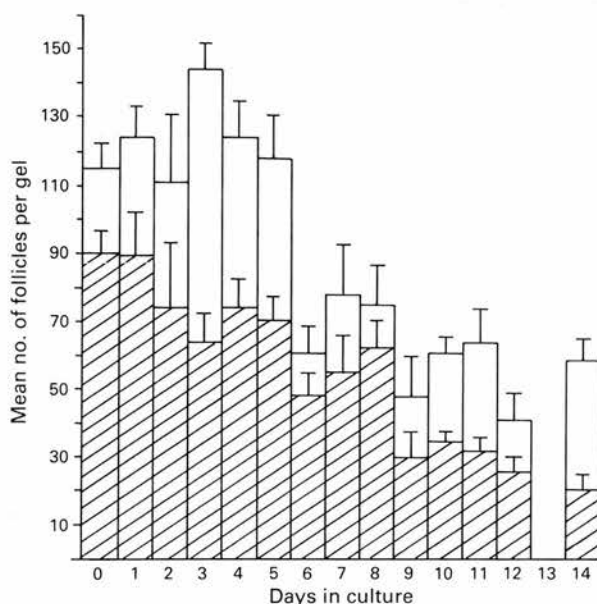


Fig. 1. A representation of the mean total number of follicles (all stages) per gel over 14 days in culture. The hatched bars represent morphologically normal follicles and the unhatched bars represent disrupted follicles.

The distribution of follicle stages underwent a marked shift with time, with small stages gradually disappearing and large ones emerging. Figure 2 shows the distribution of follicles of each stage over the course of 14 days in culture. At the start of the culture period (0 h) 43% of follicles were Stage II and 42% Stage III with only 5% being Stage IV. After a 6-day culture period the

distribution of follicle stages had altered with 36% of follicles being Stage II, 30% Stage III, 17% Stage IV and 10% Stage V (i.e. with 4 or more layers). After 14 days in culture this distribution had shifted to only 16% of follicles being Stage II, 16% Stage III, 24% Stage IV and 44% of follicles being Stage V. Stage V follicles contained 4 or more layers yet remained preantral. Antral follicles were not identified at any time and occasional spaces between granulosa cells were attributed to histological artefacts.

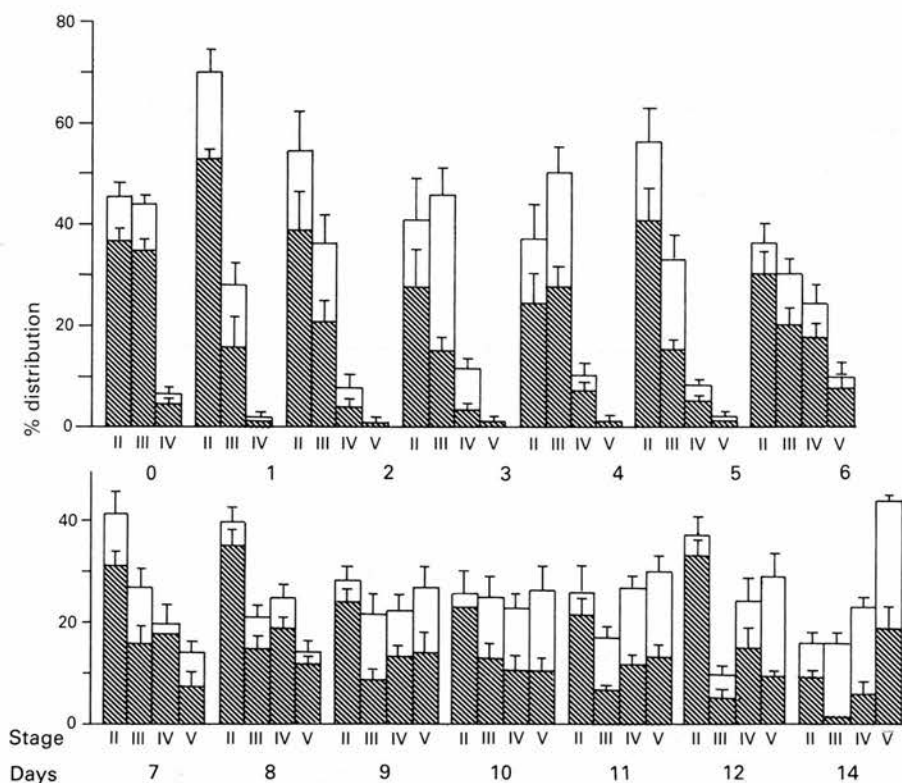


Fig. 2. Distribution of follicle stages (II-V) as a percentage of the total number of intact follicles per gel from 0 to 14 days in culture. Hatched area represents the % of intact follicles with no histological evidence of degeneration. Values are given as mean \pm s.e.m.

Between Days 6 and 8 of culture a reduction in disruption of follicle stages was found. From the start of the culture period up to Day 5 there was a gradual increase in the proportion of larger follicle types but the proportion of unhealthy follicles was high. From Day 6 the proportion of unhealthy follicles dropped and by Day 8 of the culture period 80% of follicles at all stages were histologically normal. By Day 14, however, fewer than half the follicles at any given stage were healthy.

Observation of the gels by phase-contrast microscopy during culture indicated that the follicles retained their spheroidal form, with the oocyte maintaining a central position (Fig. 3). Numerous processes were observed growing outwards from the granulosa cells. As the cultures progressed the collagen gels tended to shrink, making viewing more difficult. After several days in collagen gel culture follicles maintained an appearance (Fig. 4) indistinguishable from that in the intact ovary (Fig. 5). The major difference between the intact ovary and the cultured follicles was

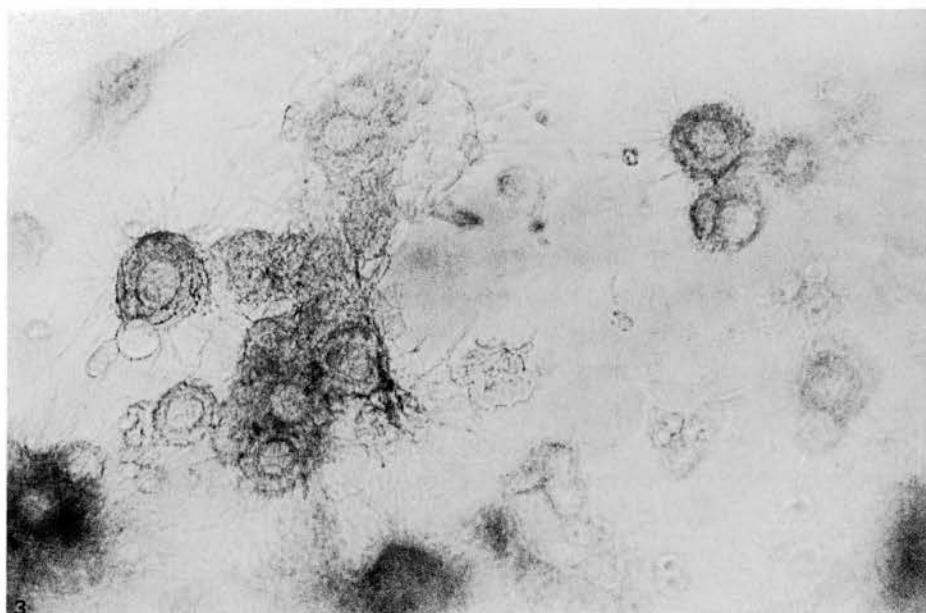


Fig. 3. Follicles in collagen gel during the culture period. The follicles maintain their structure and show cellular outgrowths. $\times 150$.

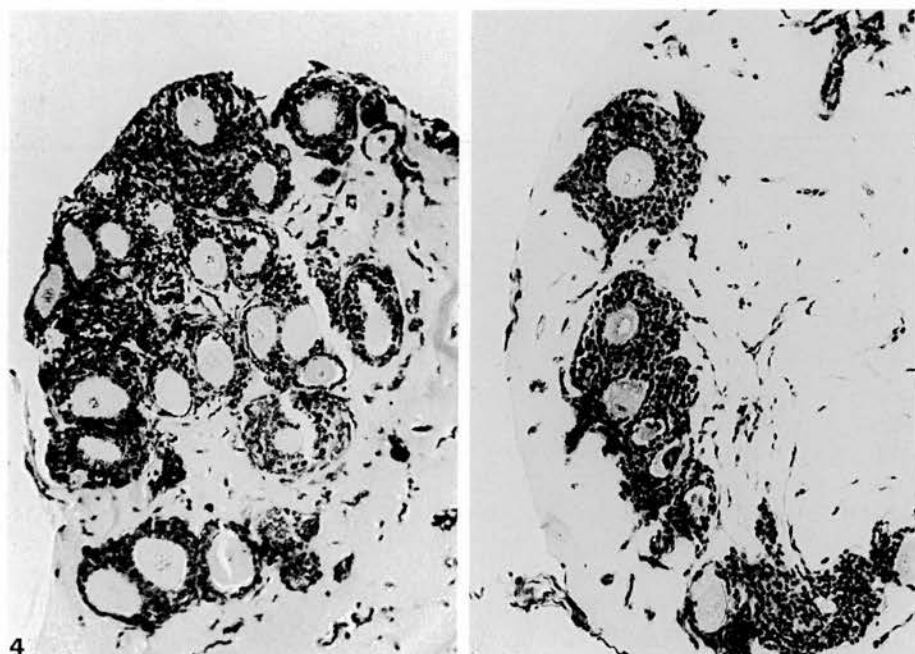


Fig. 4. Histological sections of a gel containing follicles after 6 days in culture. Unilaminar and multilaminar follicles can be seen. Some of the multilaminar follicles are showing signs of degeneration. H & E, $\times 150$.

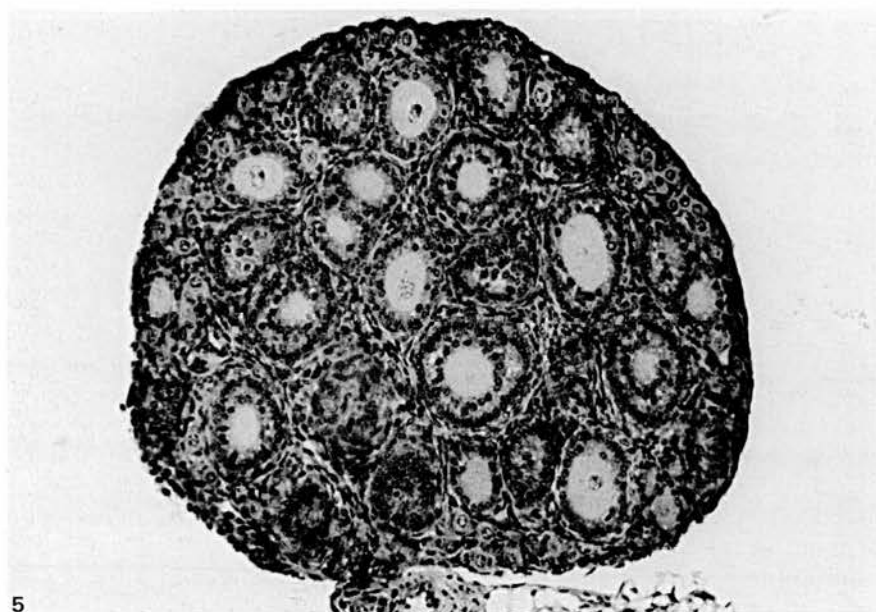


Fig. 5. Section of an intact ovary from a 10-day-old F_1 hybrid mouse. No multilaminar follicles are found in ovaries at this age. $\times 150$.

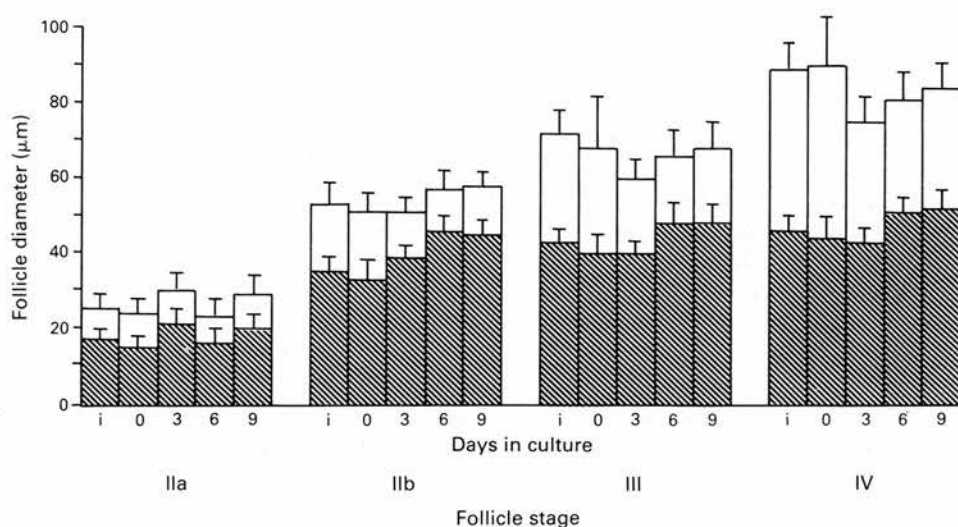


Fig. 6. Mean follicle and oocyte (hatched area) diameters at four follicle stages in intact ovaries (i) and isolated follicles up to 9 days in culture. Values are given as mean \pm s.d. for at least 50 follicles.

the presence of multilayered follicles after 6 days in culture. Stage-V follicles were absent from the 10-day-old ovary (Fig. 5) but represented 27% of the intact follicles by Day 12 in culture. Although no counts were made of primordial follicles, they were present at all times and were histologically normal.

The oocyte and follicle increased in size at each stage in culture in all groups (Fig. 6). There were no significant differences in oocyte and follicle diameter with time in the culture compared with the intact ovary, indicating that follicular development was proceeding normally.

Discussion

A technique has been developed which permits mouse ovarian follicles to be isolated and grown *in vitro* for at least 2 weeks. Using this dissociation procedure large numbers of isolated follicles can be produced. The 10-day-old mice were chosen because they do not contain Stage V-follicles (follicles with 4 or more layers). The multilaminar follicles seen after 6 days in culture have therefore emerged *in vitro* from small or even primordial follicles. Roy & Greenwald (1985) developed a similar dissociation method using collagenase and pronase to isolate the follicles of hamsters but have only carried out short-term incubations to study hormone production and receptors (Roy *et al.*, 1987). Without a gel matrix the follicular unit is disrupted but in collagen gel apparently normal growth proceeds without evidence of limiting diffusion of gases and metabolites. The collagen-gel matrix provides other advantages, i.e. a convenient method for handling follicles during histological processing and a convenient vehicle for transplanting follicles to the kidney capsule where Graafian maturation can occur (Torrance *et al.*, 1988).

These advantages of collagen gels have been exploited with several cell types, e.g. mammary epithelial cells (Yang & Nandi 1983) and thyroid epithelial cells (Chambard *et al.*, 1981). There is evidence that even with granulosa cell monolayers the purine hypoxanthine can maintain oocyte-granulosa cell complexes isolated from 10–11-day-old mice (Eppig & Downs, 1987). It would seem that hypoxanthine is maintaining functional relationships between the oocyte and granulosa cells because some oocytes are competent to be fertilized after culture but the effects of hypoxanthine on follicle units in collagen-gel culture have yet to be tested.

The culture system has been shown to be capable of supporting apparently normal follicle development up to multilaminar stages. These follicles are clearly growing from unilaminar follicles, but whether primordial follicles are beginning to grow in this system cannot as yet be determined. The relatively high proportion of unhealthy follicles present during the first few days in culture suggests that this is as a result of damaged follicles being put into culture after the dissociation. The reduction in the proportion of unhealthy follicles at all stages after 6 days in culture supports the view that follicle growth is proceeding normally. While intact small follicles often appeared identical to their *in-vivo* counterparts many of the multilaminar follicles showed signs of degeneration and the development of an antrum has not been observed in any of the cultured follicles. It would appear that the multilaminar follicles in culture had reached a stage where they might require additional support to continue growing. Media supplemented with donor calf serum were chosen to represent basal conditions as this serum contains little gonadotrophin or sex steroids but can be expected to contain other growth factors. These media may not be appropriate to support growth in later stages and we are now trying to characterize these growth requirements to obtain a chemically defined medium capable of supporting all stages of preantral follicle growth.

The gels do not only contain intact follicle units but also a variety of cells, including granulosa cells, stromal cells, ovarian surface epithelium, and other cell types. Since it would be useful to separate follicles into stages to study stage-specific effects or secretions, a method of producing a purified follicle suspension is being sought. The separation of follicles on a small scale has been achieved by using calibrated pipettes (Roy & Greenwald, 1985) but a method which would allow large numbers to be conveniently handled is required.

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Morphological study of cultured preantral ovarian follicles of mice after transplantation under the kidney capsule

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Summary. Isolated ovarian follicles taken from 10-day-old mice and cultured in collagen gel for 5 days, in the presence or absence of serum, were transplanted under the kidney capsule of ovariectomized mice. Hosts showed vaginal opening within 5 days and cornified vaginal smears by 9 days. Follicles proceeded to Graafian stages and luteinization occurred. Ovulation was not observed and oocytes degenerated within the luteinized follicle. Theca formation was preceded by the appearance of blood vessels within the graft. In-vitro fertilisation of harvested oocytes resulted in embryos.

Keywords: preantral follicles; collagen gel; culture; *in vivo*; mouse

Introduction

The follicle is the functional unit of the mammalian ovary and has a dual role—it provides the micro-environment for oocyte growth and maturation and is responsible for sex steroid production. The follicle develops from the non-growing primordial stage to a large Graafian follicle in a period of approximately 3 weeks according to age (Pedersen, 1970); about two thirds of this phase is spent at the preantral stage. We have described a method for isolating and culturing mouse preantral follicles (Torrance *et al.*, 1989) and demonstrated that ovarian follicles could develop *in vitro* from small growing stages to large multilaminar follicles. Development was blocked at multilaminar stages and neither theca layers nor antra were formed by these follicles *in vitro*. These isolated follicles may be abnormal and incapable of becoming Graafian follicles as a result of the conditions in which they were isolated and cultured. Alternatively, the culture system may be inadequate either because of the absence of signals for differentiation or the presence of inhibitors. A method was required to determine whether these follicles were capable of further development. We have now isolated and cultured follicles and transplanted them to the kidney capsule of syngeneic recipients to assess their growth and quality.

Materials and Methods

Animals. The mice used in this study were F₁ females aged 8–11 days after birth and derived from matings between CBA/Ca males and C57Bl/6 females. Adult F1 hybrid females were used as hosts for transplants. Hosts were bilaterally ovariectomized at 6 weeks of age and allowed to recover for at least 3 weeks before use. All operations were carried out under intraperitoneal tribromoethanol anaesthesia (6.03 g/kg body wt).

Follicle culture. Follicles were isolated and cultured in a collagen gel matrix as described by Torrance *et al.* (1989). The animals were killed by decapitation, the ovaries were removed and bisected before being incubated at 37°C in 1.5 mg collagenase/ml (type 1; Sigma, Poole, Dorset, UK) and 40 DNase I/ml (Sigma) for 30 min. The follicles were isolated by repeated pipetting of the bisected ovaries using Gilson pipettes. The isolated follicles were pelleted by gentle centrifugation and resuspended in 1 ml Medium 199 (Gibco, Paisley, UK) in a watchglass. Gentle rotation

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concentrated the follicles in the centre of the watchglass. Follicles were then transferred to the wells of a Terasaki plate (Flow Laboratories, Irvine, UK) and excess fluid was removed from the wells, 10 µl of the collagen gel solution were added and pipetted once to suspend the follicles. The follicles were incubated for 2–3 min at 37°C until the gel had set. Then 20 µl gel solution were pipetted into the empty wells and the 10 µl set gels were transferred to them. The double gels were allowed to set as before, then transferred to the wells of a Linbro tissue culture plate (Flow Laboratories) containing 2 ml M199 culture medium supplemented with 5% donor calf serum (Flow Laboratories). The gels were cultured at 37°C with 5% CO₂ in an humidified incubator. The medium was changed at 24 h and at 72 h.

Morphology of transplanted follicles. A total of 22 gels were obtained from 2 disaggregations using 25 and 26 animals. Follicles were cultured for 5 days and then a single gel was transferred under the kidney capsule of each host animal. Animals were killed between 2 and 21 days after treatment so that an impression of the pattern of growth over this period would be gained. Vaginal smears were taken before and after the collagen gels had been inserted. At the termination of the experiment hosts were killed by cervical dislocation and the kidney was removed. Uteri were removed from all animals and weighed. Each animal was examined to ensure that no ovarian fragments had remained after ovariectomy. The grafts were dissected and fixed overnight in aqueous Bouin's fluid, embedded in paraffin wax and sectioned at 7 µm. Sections were stained with haematoxylin and eosin and mounted with DPX. Slides were examined for the presence of Graafian follicles and theca layers.

Six ovariectomized animals received control gels and were killed after 12 days; the uteri were removed and weighed. One uterine horn from a control and one from an experimental animal were fixed in aqueous Bouin's fluid and processed for histological examination.

Follicles cultured in a defined medium. The ovaries were removed from seventeen 8–11-day-old F1 mice and follicles isolated as before. Follicles were set in collagen gel and 9 gels were formed; 3 treatment groups containing 3 gels each were established. The control group was cultured in the usual DCS-supplemented Medium 199 and a single gel was harvested at 4, 6 and 8 days. A second group was established in the control medium for 4 days and then the gels were washed with serum-free medium and transferred to a well containing Medium M199 supplemented with ITS+ (Flow Laboratories). ITS+ was used at the recommended level, 1 ml ITS+/100 ml medium, to give a final concentration of 6.25 µg insulin/ml, 6.25 µg transferrin/ml, 6.25 µg selenium/ml, 1.25 mg bovine serum albumin/ml and 5.35 µg linoleic acid/ml. Gels from this group were harvested at 6, 8 and 10 days. The third group was cultured in Medium M199 supplemented with ITS+ from the start of the culture. ITS+ and not serum was added to the gel at the polymerization stage for this group. Gels were harvested at 4, 6 and 8 days. Gels were fixed and processed for histology as above.

Transplantation of follicles cultured in a defined medium. In a second experiment the ovaries from 19 mice aged 8–11 days were used and follicles isolated as before. Six follicle-dense collagen gels were formed. The culture medium for the gels was supplemented with ITS in place of serum. Cultures of 2 gels per well were set up. Cultures were fed every 2 days. After 5 days in culture the gels were transplanted under the kidney capsule of 6 ovariectomized F1 adult hosts. The mice were examined for vaginal opening and vaginal smears were taken daily. One animal was killed 9 days after transplantation and the remaining 5 after 14 days. The grafts were removed and processed for histology. The uteri were also removed and weighed.

Observations on blood vessels and theca. Isolated follicles obtained from 25 animals were placed into 7 gels and after 5 days in culture the gels were transplanted into 7 hosts. One animal was killed on each of the days between 3 and 9 days after transplantation. Grafts were dissected and fixed in formaldehyde for 1 h followed by formol calcium overnight, embedded in paraffin wax and sectioned at 7 µm. Alternate ribbons of sections were stained with haematoxylin and eosin or with horseradish peroxidase which stains red blood cells and therefore identifies blood vessels.

In-vitro fertilization of oocytes from grafts. Follicles obtained from 14 animals were placed into 6 gels and cultured for 7 days. Gels were transferred under the kidney capsule of host animals that were ovariectomized at the time of transplantation. Hosts were injected with 10 i.u. PMSG (Organon, Lewes, Surrey, UK) 8 days after transplantation and 48 h later with 10 i.u. hCG (Organon). Animals were killed 12 h after hCG injection and the grafts were removed. Fine needles were used to separate the follicles from the gel. Oocytes were aspirated from the largest follicles and fertilized *in vitro* by established techniques (Wood *et al.*, 1987). Spermatozoa were obtained from a proven fertile F1 hybrid male. Droplets were examined 12 h later and the number of 2-cell embryos was counted.

Results

Morphology of transplanted follicles

After ovariectomy normal vaginal smear patterns were abolished and the vagina closed. The vagina began to open within 3 days of transplanting follicles under the kidney capsule and the vaginal epithelium was cornified after 7 days. Rather than presenting short regular cycles of cornification the animals exhibited persistent cornification. No vaginal opening was observed in control

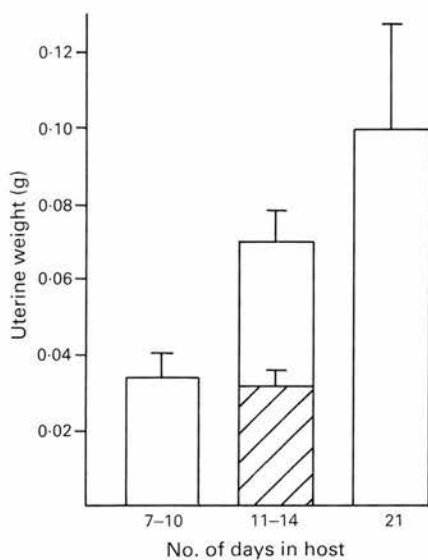


Fig. 1. Histogram showing mean \pm s.e.m. uterine weight of ovariectomized animals after transplantation of cultured follicles (open bar) or collagen gel controls (hatched bar).

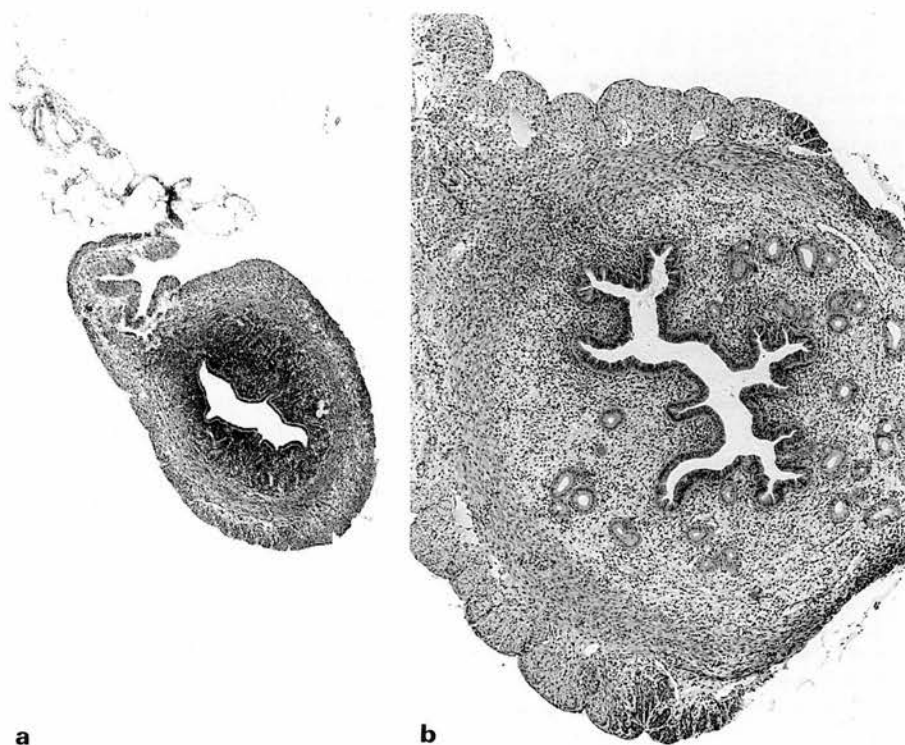


Fig. 2. Photomicrograph of uterine cross-sections from ovariectomized animals (a) 21 days after gel-only (control) transplantation and (b) 21 days after transplantation of cultured follicles under the kidney capsule.

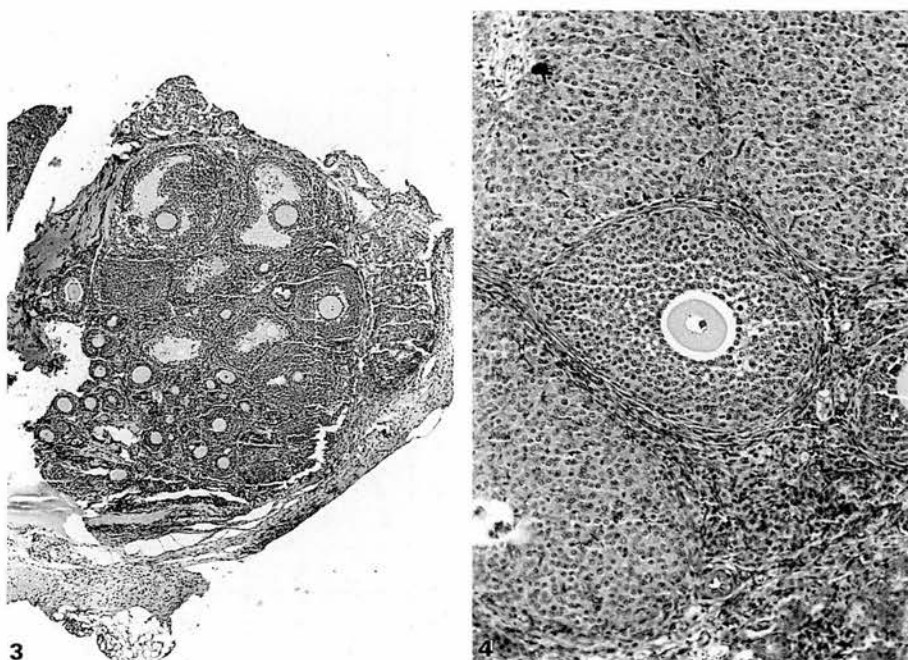


Fig. 3. Section of a collagen gel containing follicles grafted under the kidney capsule ($\times 50$). Follicles were cultured for 5 days before transplantation and the graft removed after 13 days in the host. All stages of follicular development can be seen.

Fig. 4. Section of graft showing multilaminar follicle with well defined theca layer ($\times 160$). This follicle had been cultured for 5 days and under the kidney capsule for 13 days.

animals. Uterine weights showed a significant increase compared with control animals between 11 and 14 days after transplantation (Fig. 1). The control uterus showed the expected hypotrophy of the ovariectomized state (Fig. 2a) whilst the experimental uterus showed the uterotrophic effects of oestrogen (Fig. 2b).

After the culture period and before transplantation, follicles in the gels had developed to large preantral stages but no antra or thecal development had occurred (as reported by Torrance *et al.*, 1989).

The grafts were easily identified under the kidney capsule. All stages of follicle development from primordial to Graafian were identified in the grafts from Day 7 onwards (Fig. 3). Thecal cells were observed in multilaminar follicles (Fig. 4) and large antral follicles were present (Figs 5, 6). Despite the presence of mature follicles the grafts were always anovulatory. Graafian follicles were undergoing atresia with degeneration of the oocytes within the follicles (Fig. 7) and luteinization of granulosa cells (Fig. 8).

Follicles cultured in defined medium

Microscopic assessment of the follicles from the control and experimental groups did not reveal any differences between the morphology of the follicles cultured in ITS or DCS. After 5 days in serum-free culture growth had proceeded but only to large multilaminar preantral stages. No antral follicles or follicles with a well defined theca layer were observed.

Morphology of follicles after transplantation

In the group with follicles transplanted after culture in a defined medium, vaginal opening occurred in all animals after 6 days. Vaginal smears showed evidence of cornification and uterine

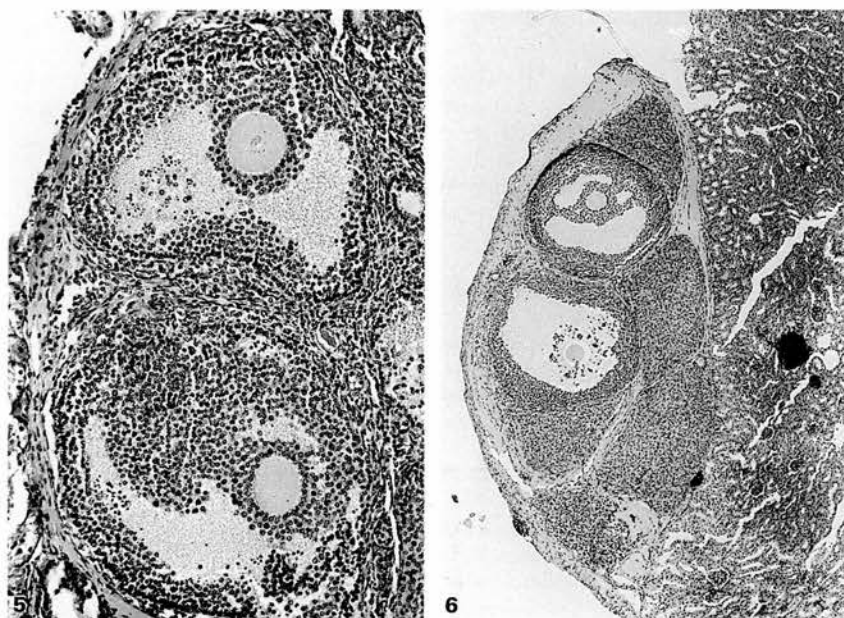


Fig. 5. Section of graft showing large Graafian follicles after 5 days in culture and 13 days under the kidney capsule $\times 160$.

Fig. 6. Section showing large antral follicle and degenerating Graafian follicle ($\times 50$). Follicles had been cultured for 5 days before transplantation and kept under the kidney capsule for 21 days.

weights increased significantly. Microscopy demonstrated the presence of a range of growing follicles including large antral follicles. These grafts were morphologically similar to those from follicles cultured in the presence of serum.

Observations on blood vessels and theca

After 3 days *in vivo* there was no evidence of theca cells or blood vessels in the graft. Some small blood vessels were apparent by 5 days but theca layers were not present around follicles. On Day 6 both theca and blood vessels were present and by 7 days both were well represented.

In-vitro fertilization of oocytes from grafts

Altogether 16 oocytes were recovered from 6 gels and fertilized and 12 developed to the 2-cell stage whilst 4 formed blastocysts.

Discussion

In this study we have shown that small preantral follicles from immature animals grown *in vitro* with or without serum are capable of progressing to the mature Graafian stage when transferred under the kidney capsule. Within 5 days these transplanted follicles were producing sufficient oestrogen to support vaginal opening in ovariectomized animals and after 7 days cornification of the vaginal epithelium was established. These results would indicate that the follicles are capable of developing beyond the multilaminar stages and that the limiting factor to further development *in vitro* appears to be the culture conditions.

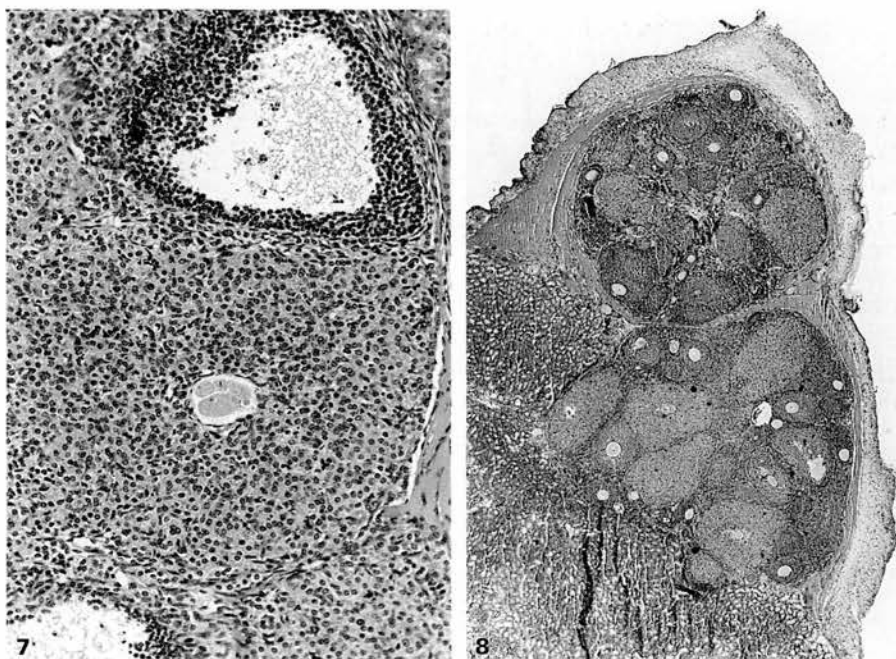


Fig. 7. Degenerating follicle with trapped oocyte ($\times 160$). Follicles were cultured for 5 days before being transferred to the kidney capsule. This graft was removed after 13 days in the host.

Fig. 8. Section of a graft showing luteal like structures ($\times 35$). Follicles were cultured for 5 days before transplantation and removed from the host after 15 days.

The results from using a partly defined medium show that follicles are capable of growing to large preantral stages without serum. The follicles appear by light microscopy to develop in a similar way to follicles in serum-supplemented media. When the follicles grown in defined medium were transplanted under the kidney capsule, they developed to large antral stages, indicating retention of their developmental potential. Follicle growth in a defined medium therefore represents a potentially powerful tool for investigating hormonal and growth factor effects on follicle development *in vitro*.

All stages of follicular development were present in the grafts. Even after 21 days *in vivo* primordial and small growing follicles of 1 and 2 granulosa cell layers were present. This suggests that the growing follicles had recently been recruited from the primordial pool. Collagenase dissociation and 5 days *in vitro* had not restricted the growth potential of the primordial follicles. The grafts appeared to remain as discrete units under the kidney capsule with no evidence of invasion by kidney cells and the grafts could be easily separated from the kidney tissue. This suggests that the differentiation of the theca and the appearance of antra were due to the endocrine environment *in vivo* and not to local interaction with the kidney cells.

In vivo thecal differentiation appears as the follicle reaches the 4 granulosa cell layer stage with antrum formation occurring at Day 13–15 (Gosden *et al.*, 1988). The presence of the theca would appear to be necessary for further follicle growth and the steroidogenic capability of the follicle is expected to be compromised when theca layers are absent (Richards & Kersey, 1979). It has been suggested that thecal cell differentiation is related to subtle increases in serum LH concentrations (Bogovich & Richards, 1982). While thecal cells were absent at the beginning of the culture, stromal cells, which are assumed to be their precursors, were present. The conditions *in vitro* were not optimum for the differentiation of stroma to theca. The results of our study show theca developing

only after the appearance of blood vessels in the graft. Endothelial cell contact or blood borne factors may be important in theca differentiation.

Ovulation did not occur from the grafts, oocytes degenerated within the follicle and granulosa cells luteinized. This pattern of follicular degeneration has been observed in the rat ovary (Popkin *et al.*, 1983) and in the mouse after suppression of ovulation by progesterone (Telfer, 1987). Intact ovarian fragments transplanted under the kidney capsule ovulate (Felicio *et al.*, 1983) and it could be that the lack of a surface epithelium in the grafts explains these differences.

Embryos were produced from oocytes taken from follicles grown *in vitro* and matured *in vivo*, showing that the culture of immature follicles does not impair their developmental potential. Using the same culture and transplantation method with frozen preantral follicles Carroll *et al.* (1989) have produced live young. Live mice have also been produced from follicles cultured under different conditions (Eppig & Schroeder, 1989).

Studies with cultures of preantral hamster follicles have shown that antral development does not occur *in vitro* unless FSH is present in the medium (Roy & Greenwald, 1989). We have conducted preliminary experiments with FSH, LH and EGF but as yet we have not observed any antral development *in vitro*. The techniques of follicle isolation and culture described in our previous paper (Torrance *et al.*, 1989) and the assessment of cultured follicles after transplantation *in vivo* provide the opportunity to study the factors influencing the development of follicles from the primordial to preantral stage. With further development of the techniques it may be possible to produce all stages of follicular development *in vitro*.

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Physiological factors underlying the formation of ovarian follicular fluid

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Introduction

In common with all other cells, the oocyte and granulosa cells are bathed in extracellular fluid. It has, however, become conventional to reserve the term 'follicular fluid' for that fraction of the extracellular fluid that accumulates in the antrum of larger follicles. This pool of fluid is of considerable biological significance since its composition indicates the environment in which the oocyte and granulosa cells are growing and maturing. Furthermore, it buffers the internal environment of the follicle against the influence of external conditions presented by the blood stream.

The chemical composition of follicular fluid has been studied extensively and found to consist of substances derived from blood as well as from local secretion and metabolism. Particular attention has been paid to the proteins and hormonal steroids. Rather than attempt a comprehensive review, this paper will focus on general physical characteristics of the fluid and the physiological factors that influence its formation. These properties determine the rate at which extracellular fluid is accumulating and, hence, the size and morphogenesis of the Graafian follicle. It is important to reveal the mechanism and dynamics of follicular fluid formation if the composition of the fluid is to be fully understood.

Ontogenesis of the follicular antrum

Follicles do not possess a major pool of extracellular fluid from the beginning of their development. The primordial follicle consists of a relatively small oocyte surrounded by a single layer of squamous cells which are destined to become the granulosa cell layer (membrana granulosa). Pools of follicular fluid appear when the granulosa cells have passed through about 11–12 mitotic cycles and a solid follicle containing 2000–3000 cells and with a diameter of 150–400 μm has been built. These pools coalesce to form a single spherical cavity, the antrum, which is central and bounded by a layer of granulosa cells of uniform thickness except at the pole where cumulus cells are attached. The antrum characterizes the mature follicles of most mammals but it is not universally found. Ovulation is said to occur from 'solid' follicles in a number of insectivores (Mossman & Duke, 1973). These ovulations occur precociously because the follicles scarcely exceed the dimensions at which the antrum would normally form.

Quantitative aspects of follicular fluid formation

There is evidence which suggests that the size of follicles at the time of antrum formation varies with body size (Parkes, 1932; McNatty, 1978) although the raw data show that variation within a species is of a magnitude similar to that between species. There can be no doubt however that the

size of follicles shortly before ovulation varies with body size. When the volume of a preovulatory set of follicles is plotted against body weight on logarithmic axes, an isometric relationship is obtained (Gosden & Telfer, 1987). Most of this volume is extracellular space except in a few species in which the small follicles contain a diminutive antrum (e.g. *Sorex araneus*). Body size is not the sole arbiter of Graafian follicle volume since relatively small follicles occur in species that release prodigious numbers of eggs, although the collective volume remains commensurate with body weight (Weir, 1971). The size of the mature Graafian follicle is genetically determined and is of physiological significance. The volume of the antrum will influence the concentration of hormones and metabolites in the extracellular fluid and, consequently, their biological actions. Formation of the antrum requires the combined actions of FSH and oestrogen (Goldenberg *et al.*, 1972) and it is anticipated that the volume changes will also be regulated by polypeptide and/or steroid hormones. In this regard, it may be significant that the concentrations of oestrogen and androgen in the antrum are very different in follicles that are expanding compared with those that are shrinking (atretic) (McNatty *et al.*, 1979).

After Robinson (1918), it has been customary to distinguish three phases of follicular fluid formation. Primary fluid ("liquor") is produced until shortly before ovulation when the rate of accumulation rises abruptly. Secondary fluid is produced at this time, and presumably under the influence of the surge of gonadotrophic hormones. Tertiary fluid is produced in the collapsed follicle after ovulation. Robinson regarded primary fluid as having an intracellular origin whereas secondary fluid has been attributed to transudation from the thecal capillaries, a conclusion which has been upheld by many later commentators. Much less attention has been given to tertiary fluid; it will not be considered further here.

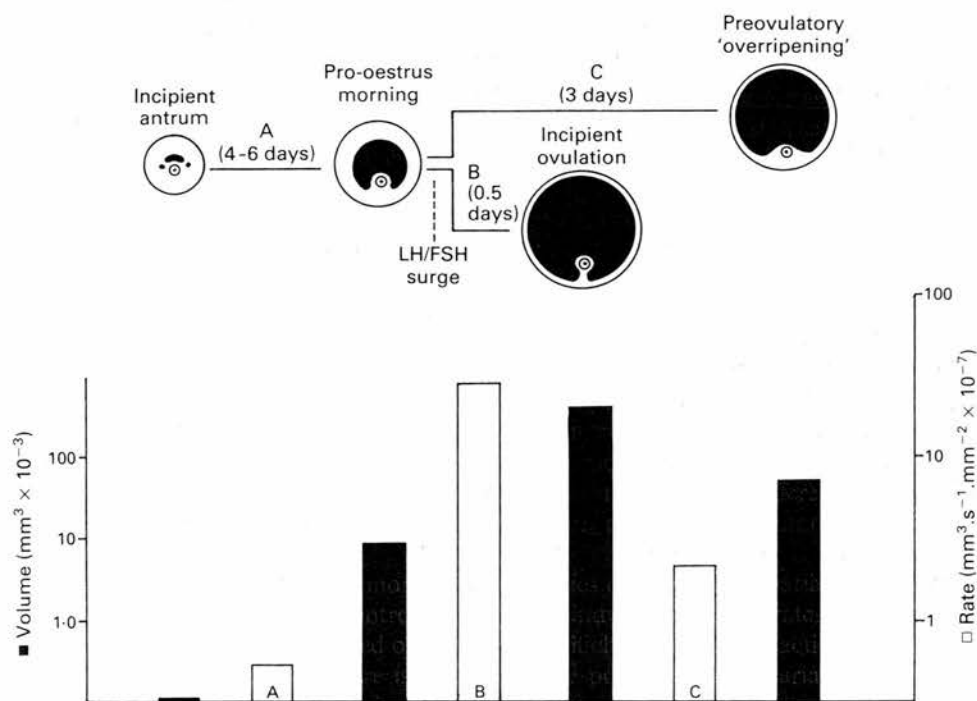


Fig. 1. The upper panel depicts the expansion of the antrum in mouse follicles which are either proceeding to ovulation (A–B) or, in the absence of an ovulatory stimulus, to formation of a follicular cyst (A–C). The lower panel represents the volume of the antrum at different stages and the estimated rates of fluid accumulation expressed on logarithmic axes. Measurements are based on histological sections and assume linear rates of growth.

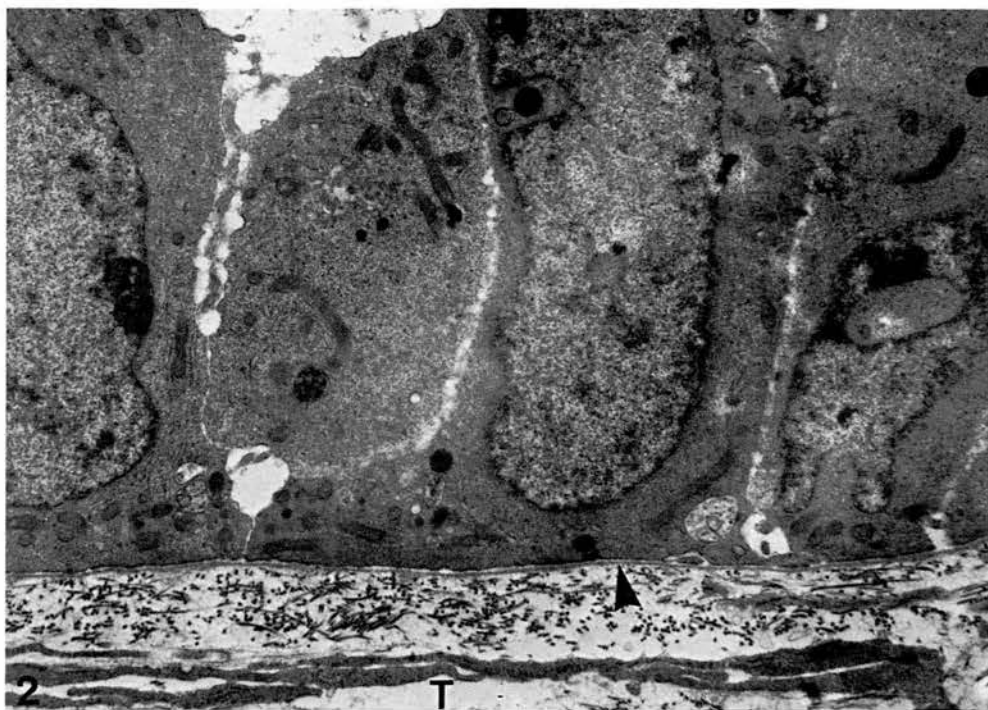


Fig. 2. The ultrastructural appearance of the peripheral layer of granulosa cells which rest on a basement membrane (arrow) in this rabbit follicle. The vascular theca (T) lies on the opposite side of this membrane. $\times 9000$.

In view of the long-established distinction between primary and secondary fluids it is surprising that this has not been quantified. The volume of the antrum and rate at which it is expanding can be estimated simply by making measurements of tissue sections. It is assumed that artefactual shrinkage is a minimal and constant factor and that the rate of expansion is more-or-less constant at each phase. Results which were obtained from mouse ovaries have verified the distinction between the fluids (Fig. 1). When standardized for differences in surface area, the results indicate that the rate of extracellular fluid formation during preovulatory swelling rises about 50-fold above that of the previous phase. When the gonadotrophin surge is lacking, swelling continues (Laing *et al.*, 1984), but it is at a slower rate than that of follicles undergoing preovulatory activation, strengthening suspicion that hormones stimulate this process (Fig. 1). Antral follicles take much longer to grow to full size in large species than in small ones. Calculations based on data obtained by Turnbull *et al.* (1977) and Driancourt *et al.* (1986) for the sheep indicate that a distinction can be made between the rates of primary and secondary fluid formation, these being of the same order of magnitude as in mice. By comparison with the secretion/absorption of fluids across many other membranes, the rate of follicle swelling is sluggish, adding to the practical difficulties of investigating its formation. In, for example, the anterior chamber of the eye, aqueous humour is produced at a rate of about $2 \mu\text{l} \cdot \text{min}^{-1}$ whereas the corresponding value for ovine follicular fluid during the preovulatory phase is $20 \mu\text{l} \cdot \text{day}^{-1}$.

The membrana granulosa: an epithelial layer

Epithelial cells either line cavities or cover surfaces of the body and, as a consequence of their location, can regulate the internal environment of body compartments. These cells are therefore

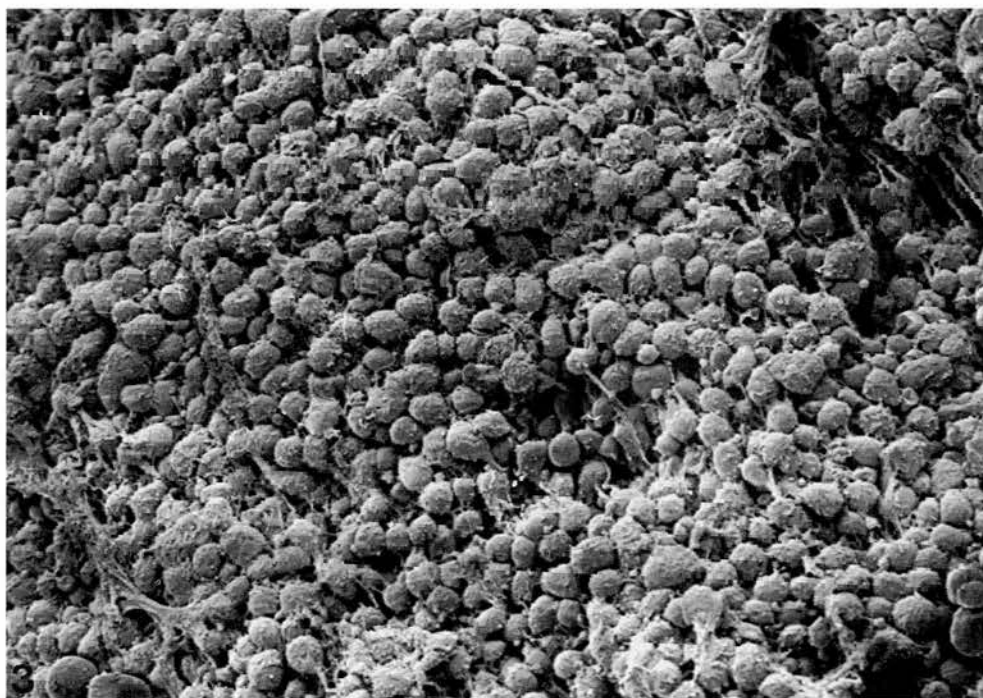


Fig. 3. Scanning electron micrograph of the inner surface of the mural epithelium of a sheep Graafian follicle. The granulosa cells present a regular mosaic with relatively smooth surfaces. No large channels are visible opening into the antrum. $\times 2025$.

expected to be specialized for promoting/restricting the movements of particular ions and molecules between compartments. Since granulosa cells are an epithelial type, they may be expected to exhibit these functions. The granulosa cells of growing follicles are typically polygonal although the outer layer is columnar and rests on a delicate basement membrane (Fig. 2). Up to about 10 cell layers develop in preantral follicles but these attenuate when the antrum expands because the follicle wall is compliant and mitotic activity has virtually ceased. The epithelial surface facing the antrum is relatively featureless: it is uniform and lacking in conspicuous channels or processes (Fig. 3). The granulosa layer is the same thickness throughout except at the pole containing the cumulus oophorus in which cellular proliferation continues (Gosden *et al.*, 1983).

The morphology of the follicular epithelium suggests a structure that is highly permeable to water and dissolved substances. In contrast to so-called 'tight' epithelia (e.g. frog skin, toad bladder, trophoderm), occlusive junctions are not found between cells, although other junctional complexes exist for maintaining structural integrity and intercellular communication (Albertini & Anderson, 1974; Fig. 4). Granulosa cells are separated by channels measuring 20 nm which permit molecules of up to M_r 500 000 in size to penetrate and reach the antrum (Zachariae, 1958; Albertini & Anderson, 1974; Payer, 1975; Cran *et al.*, 1976). The density of cell packing decreases centripetally and is further reduced in the cumulus cells during mucification. Thus, the extracellular fluid of the follicle is a continuum and sub-compartments are not well-defined by structural barriers. Hence, many potentially toxic substances carried in blood can reach the oocyte (Baukloh *et al.*, 1985).

Small spherical spaces containing extracellular fluid appear in the granulosa layer of some species when the antrum is incipient. The significance of these Call-Exner bodies is not known although it has been suggested that they are sites of active secretion of follicular fluid (Brambell, 1956) and may be related to the enlargement of the Golgi apparatus (Hadek, 1963). A major

role is doubtful because they remain separate from pools of primary follicular fluid and exist independently of an antrum in human granulosa cell tumours.

Chemical composition of follicular fluid

Chemical studies of secondary fluid have been encouraged by accessibility in large follicles. There is, however, a paucity of information about primary fluid in small follicles (< 1 mm) and the extent to which the composition of these fluids differs remains unclear. Particular attention is being paid here to electrolytes since the organic components of follicular fluid have been reviewed thoroughly elsewhere (Edwards, 1974; McNatty, 1978; Lenton, 1988).

Table 1 shows the concentrations of the principal electrolytes in large (mainly preovulatory) follicles of 5 species together with the values for plasma/serum. No major concentration gradient exists across the follicle wall. There is tentative evidence, particularly from smaller follicles, that the concentrations of K^+ are greater in follicular fluid than in blood. This interesting finding parallels the situation in the oviduct (Borland *et al.*, 1980) and could indicate active inward transport of the cation. It requires verification because the possibilities that either K^+ had leaked from damaged cells or that atretic follicles had been sampled were not excluded. Furthermore, any results obtained with fluids obtained *post mortem* must be considered unreliable because of the rapid changes that take place (Edwards, 1974; Knudsen *et al.*, 1978). In view of variable sample quality, earlier claims that electrolyte concentrations during the ovarian cycle and at different stages of follicle development require re-examination. Measurements of Na^+ are probably more reliable than those of K^+ because this cation is abundant in extracellular fluid but scarce in cells. The slight, but significant, elevation

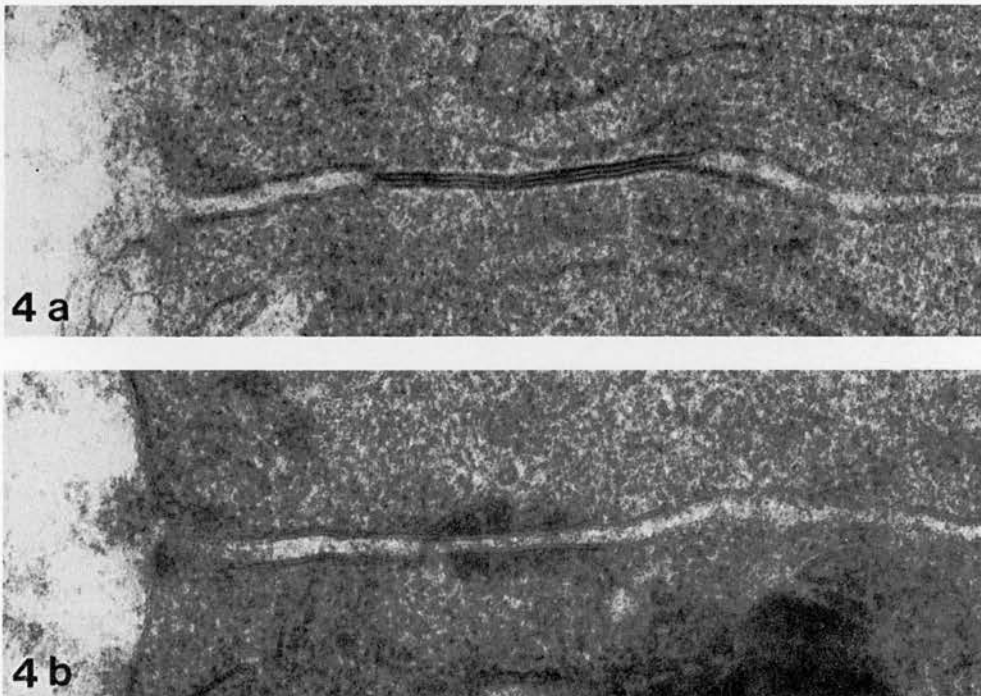


Fig. 4. Channels between adjacent granulosa cells are visible in this rabbit Graafian follicle. The cells are connected by a junctional apparatus resembling (a) gap junctions and (b) maculae adherens. $\times 90\,000$.

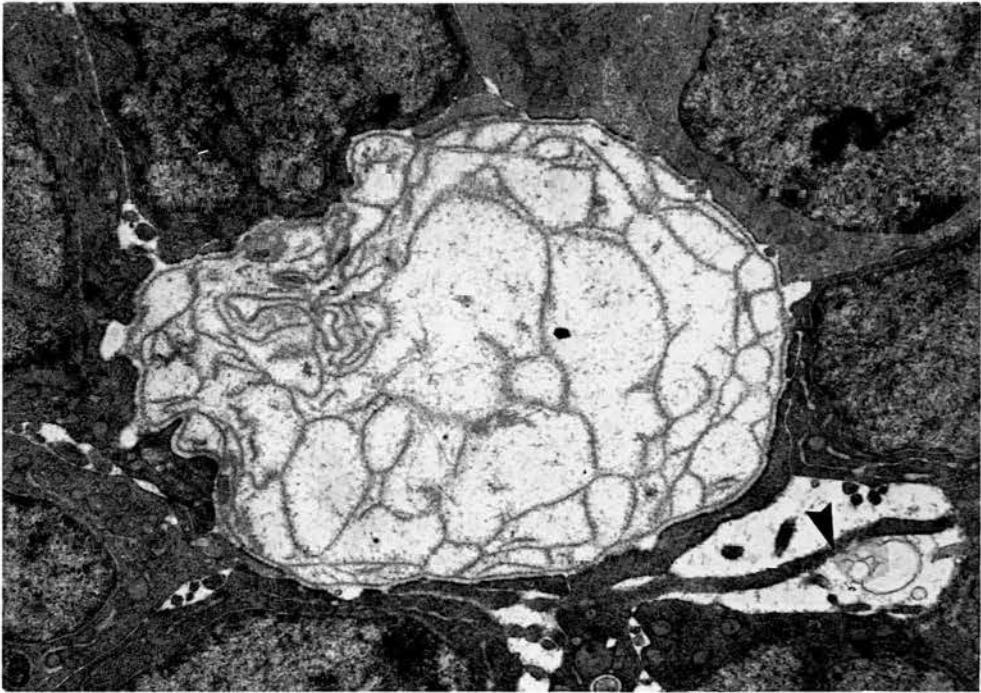


Fig. 5. A Call-Exner body situated in the wall of a rabbit Graafian follicle. These structures frequently contain long strands of moderately electron dense material suspended in the extra-cellular fluid and in apposition to the epithelial cells. The apical faces of these cells are smooth although there are long processes in the vicinity (arrow). $\times 10\,000$.

Table 1. Electrolyte concentrations (mmol. l^{-1}) in the fluids of large follicles (FF) as compared with plasma (P) or serum (S)

Species	Na ⁺		K ⁺		Cl ⁻		Ca ²⁺		Mg ²⁺		Reference
	FF	P/S	FF	P/S	FF	P/S	FF	P/S	FF	P/S	
Human	124	145	4.4	4.6	109	104					Shalgi <i>et al.</i> (1972) Chong <i>et al.</i> (1977)
	143	154	5.4	5.4	140	146	0.94	1.04	0.76	0.68	
Rabbit	133	125	7.0	4.3	136	127					David <i>et al.</i> (1973) Burgoyne <i>et al.</i> (1979)
	140	136	6.2	5.7	144	139	3.56	3.85	1.40	1.67	
Sheep	149	149	4.7	4.9	107	106	2.29	2.28	0.89	0.87	R.G. Gosden & R.H.F. Hunter (unpublished)
Pig	128	143	15.9	5.2							Schuetz & Anisowicz (1974)*
	142	147	7.6	7.1			10.3	10.8			Chang <i>et al.</i> (1976)*
	145	140	4.9	4.8							Knudsen <i>et al.</i> (1979)
	141	138	3.8	3.8	97.3	95.7	2.30	2.27	0.75	0.77	R.G. Gosden & R.H.F. Hunter (unpublished)
Cow	132		9.2		149.5		3.1				Olds & VanDemark (1957)*

*Follicular fluids were obtained *post mortem*.

of Na^+ in follicular fluid of some species (e.g. pig, rabbit), although not all (e.g. sheep), may result from active transport processes (see below). No concentration gradients have been identified for the other ions (Cl^- , Ca^{2+} , Mg^{2+}).

These results together with those obtained with many small organic molecules strengthen the conclusion that most substances can diffuse freely into or out of the follicle. This does not need to imply, however, that equilibrium conditions exist; indeed, this is doubtful since the follicle is never static because of continuous changes in volume and metabolism.

The protein concentrations have attracted most attention because hormones have to gain entry to the follicle to influence the granulosa cells. *In toto* they range from 50 to 100% of normal plasma values but they are not present in equimolar proportions (McNatty, 1978). Larger proteins penetrate more slowly than small ones and the concentrations present at a particular stage can be expected to vary with the flux of water, the surface area to volume ratio of the follicle and the permeability of the thecal blood vessels. Follicular fluid contains most of the plasma proteins, albumin being the most abundant in both cases. Plasma proteins of $M_r \geq 850\,000$ are absent (Shalgi *et al.*, 1973) and some large molecules produced by granulosa cells (e.g. proteoglycans) probably do not escape from the follicle (Ax & Ryan, 1979). The follicular wall therefore behaves like a coarse molecular sieve and a blood-follicle barrier exists. The gonadotrophins, FSH and LH, are not excluded by this barrier, although present at lower concentrations than in blood (McNatty *et al.*, 1975).

Respiratory gases and acid-base balance

Oocytes are isolated from the capillary circulation because the follicular epithelium is avascular. As a consequence of diffusion through layers of respiring cells, the concentration of oxygen around the oocyte is expected to be attenuated to an extent which will vary with the size and form of the follicle. A steeply descending inward concentration gradient has been predicted for large preantral follicles by a simple mathematical model (Gosden & Byatt-Smith, 1986) but Graafian stages are not so amenable to modelling because of their multicompartmental structure and asymmetry. Until direct measurements of the P_{O_2} can be made, inferences will have to be drawn from the analysis of follicular fluid whilst recognizing that these values could be substantially different from those of the local environment of the oocyte.

Table 2. The composition of follicular fluid with respect to blood gases, bicarbonate and pH

Species	P_{O_2} (mmHg)	P_{CO_2} (mmHg)	HCO_3^- (mmol. l^{-1})	pH	Reference
Human	54.3	35.1	—	7.27	Shalgi <i>et al.</i> (1972)
Human	103.5	43.2	—	7.34	Fraser <i>et al.</i> (1973)
Pig	51	45	28.0	7.41	Knudsen <i>et al.</i> (1978)
Pig	—	52.1	27.3	7.34	R.G. Gosden & R.H.F. Hunter (unpublished)

Respiratory gas tensions in follicular fluid have seldom been measured and the available data are highly variable (Table 2). Much of this variability is probably artefactual because there are several sources of potential error. The data are, however, sufficiently consistent to conclude that the fluid is not anoxic and the P_{O_2} may even be close to that of normal ovarian venous blood. Clarification is required. Measurements of the P_{CO_2} and pH are within the venous range and more consistent. Since follicular fluid has a similar composition to plasma, the pH will be buffered by carbonic

acid and protein (Shalgi *et al.*, 1972). The pH of the extracellular fluid, which is 7.3–7.4 in the antrum, may differ locally as a result of differences in metabolism and secretion, e.g. hyaluronic acid and chondroitin sulphuric acid.

Physiology of follicular fluid formation

The morphological and chemical evidence discussed so far leads to the expectation that most molecules will move freely across a porous follicular wall according to their concentration gradient. They strengthen the early view that transudation from capillaries is responsible for secondary (and perhaps other) follicular fluid (Robinson, 1918; Burr & Davies, 1951). The possibility remains, however, that more than one mechanism exists for the transport of fluid, as is the case in other 'leaky' epithelia. In the gall bladder active outward transport of salt is followed by the net movement of water down its osmotic gradient (Spring & Ericson, 1982). A similar mechanism, perhaps controlled by hormones, could explain the formation of follicular fluid provided that the polarity of salt transport was reversed. An alternative suggestion has been made that hydrolysis of polymeric glycosaminoglycans in the antrum could raise the osmotic potential and cause follicles to swell, leading to ovulation (Zachariae, 1957; Zachariae & Jensen, 1958). Either hypothesis would explain why the shrinkage of the antrum and pycnosis of mural granulosa cells occur concurrently in atretic follicles. The latter one is no longer favoured, however, because the colloid osmotic pressure is not elevated during the preovulatory phase and ovulation occurs without a raised intrafollicular pressure (see below). The question of whether salt transport is involved has not been answered. It appears that follicles possess osmotic properties because they shrink when immersed in hypertonic saline, demonstrating that the epithelium is sufficiently semipermeable.

The rate and direction of net water movement between the two compartments, namely plasma (strictly interstitial fluid/lymph) and follicular fluid, depends on the magnitude and sign of their chemical potentials. In the absence of temperature or hydrostatic pressure gradients these potentials are predicted by the depression of the freezing point of the fluids. The extracellular fluids of the sheep ovary are isotonic, with an osmotic pressure equivalent of 300 mosmol.kg⁻¹ (Table 3). Whilst this result appears to deny that water enters the follicle as an osmotic consequence of secretion of solutes into the antrum, there is evidence that gradients <1 mosmol.kg⁻¹ can produce substantial water transport (Spring & Ericson, 1982). In view of the slow accumulation of follicular fluid, particularly at early stages, it is not justifiable to dismiss the active transport hypothesis.

Table 3. Freezing point depression (°C) of ovarian fluids from sheep measured using a modified Ramsay–Brown apparatus (Ramsay & Brown, 1955) using a quartz crystal thermometer sensitive to 1/1000°C (unpublished data)

Ovarian venous plasma	Ovarian lymph	Follicular fluid	
		Follicle >6 mm diam.	Follicle 2–6 mm diam.
-0.557 ± 0.006	-0.587 ± 0.021	-0.548 ± 0.005	-0.551 ± 0.005

Values are mean ± s.e.m. for 7–11 observations.

If an osmotic gradient is being considered the Na ion is the prime candidate for active transport because of its abundance and the established role of the Na,K-ATPase in water transport in some epithelia. This suggestion is supported by the slight excess of Na in the antral fluid of some species and the binding of ouabain to granulosa cell membranes (R. G. Gosden, unpublished). The fact that granulosa cell monolayers do not form domes of secreted fluid, as do other transporting

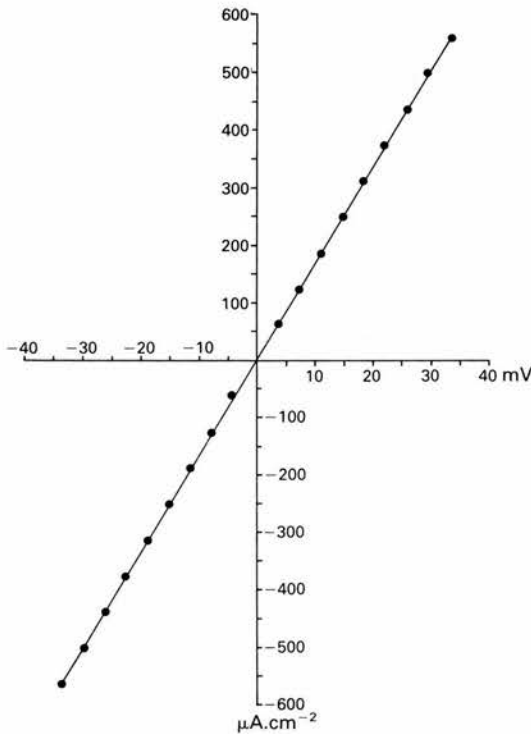


Fig. 6. The relationship between current and voltage in the pig follicle wall as obtained in a voltage clamp experiment (for method see Civan, 1983). The current required to maintain a predetermined voltage was measured and the transmembrane resistance has been calculated using Ohm's Law (see Table 4). The open circuit voltage and short-circuit current are found at the intersections between the line and the x and y co-ordinates, respectively: these are close to zero in this structure.

epithelia (Widdicombe *et al.*, 1987), should not be regarded as contradictory evidence because the direction of transport is the opposite of that of absorptive epithelia.

The movement of ions across a membrane can establish a transmembrane potential difference which can be measured electrophysiologically to provide evidence of active transport. When this voltage is electronically clamped to zero the current passing ('short-circuit current') indicates the direction and net movement of charged particles which can then be identified by inhibiting transport mechanisms (Ussing & Zerahn, 1951; Civan, 1983). This method has become conventional for studying transport processes in epithelia and has now been applied to the isolated follicle wall of the pig. The open-circuit potential difference (i.e. unclamped) was found to range from $+0.5$ to -0.5 mV and the short-circuit current was close to zero (Fig. 6). These data provide no support for the active transport hypothesis, but nor do they deny it. McCaig (1980, 1985) has tackled the same question by the alternative approach of measuring the potential difference of superfused mouse follicles using microelectrodes. As the microelectrode advanced, voltage changes were encountered: firstly as surface epithelial and granulosa cells were traversed successively, in which the membrane potentials were -21.9 ± 0.4 mV, and finally, as the potential difference fell to $+1.2 \pm 0.3$ mV, when the antrum was entered (Fig. 7). This small antral potential difference became more positive near the time of ovulation or after treatment with metabolic inhibitors. Since the resistance did not change concomitantly it was inferred that active transport was responsible for the difference.

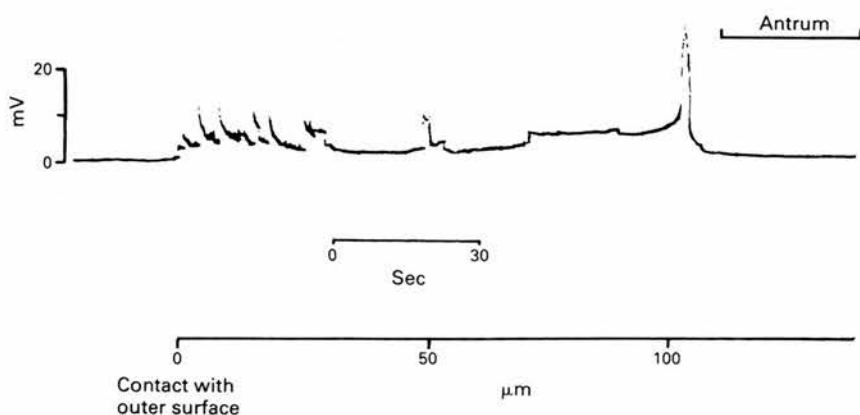


Fig. 7. Electrical potentials recorded using a glass microelectrode traversing the wall of a mouse follicle and entering the antrum. A number of intracellular potentials (negative-going, upward deflections) are recorded before a steady, slightly positive potential is reached in the antrum (McCaig, 1980).

Since there are transmural differences in the concentrations of relatively impermeable charged molecules (proteins and glycosaminoglycans) conditions favour the existence of a Gibbs–Donnan equilibrium. The small (if significant) transmural potential differences could therefore have developed merely from diffusion of the major ions. The equilibrium potentials for these ions can be calculated from their concentrations on either side of the follicle wall using the Nernst equation assuming that the activity coefficients are the same in the two fluids (Borland *et al.*, 1977). The potentials corresponding to our data from the pig are: Na, -0.69 ± 0.05 mV, K, $+0.32 \pm 0.16$ mV, Cl, -0.40 ± 0.47 mV. These are consistent with the measured electrical potentials. They do not, however, rule out active transport because leaky membranes are less able to hold a charge. Definitive testing of active transport will require direct measurement of Na^+ and Cl^- fluxes.

The specific electrical resistance of the follicle wall can be calculated from the relationship between the transmural potential difference and the current being passed (Fig. 6). This relationship is linear which indicates that the follicle wall behaves as a simple ohmic conductor without significant rectification. It is concluded that the current is probably conducted by a paracellular rather than

Table 4. Comparison of electrical properties of the ovarian follicular wall with those of other mammalian epithelial cells

Epithelium	Species	Classification	Specific resistance (Ωcm^2)	Reference
Ovarian follicle (unstripped)	Mouse	Leaky	49	McCaig (1980)
	Pig		60	R.G. Gosden & R.H.F. Hunt (unpublished)
Trophoblast	Rabbit	Tight	2650	Cross (1973)
Proximal convoluted tubule	Dog	Leaky	6	Boulpaep & Seely (1971)
Ileum (unstripped)	Rabbit	Leaky	89	Field <i>et al.</i> (1971)
Gall bladder	Rabbit	Leaky	28	Frömter & Diamond (1972)

paracellular route, which is consistent with the evidence that hydrated channels exist between the cells. The follicle wall resistance is sufficiently low that the epithelium can be classified with those that are electrically 'leaky' (Table 4). This resistance is in fact the sum of the resistance of the ovarian surface epithelium and the follicle itself, of which the latter may be the minor component.

Up to this point it has been assumed for the sake of argument that the total number of particles present (i.e. osmotic potential) determines the rate and direction of net water transport. Chemical potential is, however, influenced by differences in temperature and hydrostatic pressure (Patton, 1965). Whilst it might not be expected *a priori* that the temperature inside follicles would be different from that of the body core, the surprising claim has been made that rabbit follicles have a temperature -3°C lower than the ovarian stroma (Grinstead *et al.*, 1980). If substantiated, this finding adds another, albeit minor, factor to the list of those responsible for the formation of follicular fluid.

Finally, it is necessary to discuss critically the evidence for transudation. The follicle wall is very permeable to water (Peckham & Kiekhof, 1959) and although the hydraulic conductivity of granulosa cells has never been measured, it is assumed that most water enters is paracellularly. Transudation requires a hydrostatic pressure gradient from capillary to antrum although the pressures have been found to be similar (~ 17 mmHg) (Blandau & Rumery, 1963; Espey & Lipner, 1963; Rondell, 1964). Accurate measurement under physiological conditions is difficult and small, undetectable gradients could still account for the slow accumulation of follicular water. According to this hypothesis, a change in interstitial pressure, perhaps under the influence of hormones, would lead to swelling of the follicle which would bulge into the lower pressure region of the ovarian bursa or peritoneal cavity, as occurs when the ovarian vein is experimentally clamped to raise intravascular pressure. There is evidence of stromal oedema and increasing leakiness of the thecal capillaries during the periovulatory period (Morris & Sass, 1966; Byskov, 1969; Bjersing & Rajander, 1974), but the existence of pressure gradients remains to be demonstrated.

Transudation provides the most satisfactory explanation for the formation of secondary follicular fluid which is occurring relatively rapidly. It might be argued, however, that it is an unwieldy force for the morphogenesis of the antral follicle and for the controlled production of primary fluid. That other mechanisms are involved at early stages of development may be inferred from studies of mouse ovaries in organ culture in which follicles were found to undergo formation and limited expansion of the antrum in the presence of FSH (Ryle, 1969). Since a vascular supply was absent it seems likely that antrum formation requires active secretion. It is not clear whether any secondary fluid can be produced under similar conditions although this appears to be doubtful because rat ovaries ovulating *in vitro* did not undergo a normal increase in weight (Osman & Liewwama-Boordanus, 1980).

Throughout much of this review, there has been an assumption that follicular fluid is homogeneous whereas, in reality, microenvironments undoubtedly exist. Not only would these be anticipated in the vicinity of the oocyte and its investment of cumulus cells but also within the mural granulosa cell layers as a result of local metabolism/secretion and fluxes across the follicle wall. And even within the antrum unstirred layers may be encouraged by mucification. A major focus of future research should therefore be the charting of these differences.

Conclusions

The composition of follicular fluid in Graafian follicles is similar but not identical to ovarian venous plasma. Differences between the two fluids are attributed to a blood-follicle barrier, which restricts the passage of large molecules, and to the existence of a hypothetical active transport mechanism and/or a Donnan equilibrium, reflected by the distribution of small permeant ions. The follicle epithelium has been characterized as 'leaky' on the basis of both chemical and electrical criteria.

The rate of follicular fluid accumulation is much greater during preovulatory activation of the follicle (secondary fluid) than at preceding stages (primary fluid), suggesting that gonadotrophic hormones have a major influence on the rate of swelling. The evidence for water transport following an osmotic gradient set up by active transport of Na^+ has been inconclusive. The conventional view that fluid forms from transudation of plasma rests on circumstantial evidence and is less likely to account for primary than for secondary fluid. The discussion has focussed on the fluid in the antrum principally because so little is known about the fluids which lie in the narrow extracellular spaces and bathe the granulosa cells and oocyte. A major challenge for future research will be the elucidation of the compositions of these microenvironments which, in contrast to the bulky antral fluid which will buffer change, should be sensitive indicators of fluctuating biosynthetic activity and of optimal conditions for cell culture.

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